THE EFFECT OF DIETHYLSTILBESTROL (DES) TREATMENT ON THE ESTROGEN TITER OF THE MATERNAL, FETAL AND NEONATAL OVARIAN TISSUE OF THE LONG-EVANS RATS

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ABSTRACT

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The Effects of Diethylstilbestrol (DES) Treatment on the Estrogen Titer of the Maternal, Fetal and Neonatal Ovarian Tissue of Long-Evans Rats

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Diethylstilbestrol (DES) is one of the synthetic estrogens available today for therapeutic use. It is also referred to as a carcinogenic agent, with a great number of side effects reported between maternal ingestion of DES during pregnancy, as a postovulatory agent to prevent implantation and possible occurrence of carcinoma in the progeny of women known to be aborters. In the present study, experiments were done to determine the effects of DES on the estrogen titer of the maternal, fetal and neo-natal plasma ovarian homogenates of female Long-Evans rats.

Adult and 30-day old rats were treated via stomach intubation with 35 mg/2cc/kg body wt of DES. The estrous cycle of the rats was monitored and vaginal smear cell counts were determined in 0.5 cc saline smear volume. Radioimmunoassay was used to determine the estradiol levels in the plasma and ovarian homogenates.

Results showed no changes in the estrous cycle synchrony of the

the maternal rats, while the estrous cycle of the treated 30-day-old rats was synchronous. The vaginal smear cell count was significantly greater in the two groups of treated rats studied than in the controls. Comparing the mean estradrol value for the plasma and ovarian homogenates in the maternal rats, an obvious increase in the plasma E_2 level was obtained as compared to small decrease in the mean value of ovarian E_2 level. There was no difference in the mean value of pooled ovarian homogenate E_2 level of the control saline, control and DES-treated 19-1/2-day old rats. A significant decrease in the plasma E_2 mean value of 30-day old treated rats was obtained when compared to both control groups. There was no significant increase in ovarian homogenate E_2 level of both treated and the control groups.

In light of these results, DES has a secondary effect, asynchronous cell proliferation in the vaginal epithelia leading to prolonged specific stages of the estrous cycle of the young adult female rats. Also, DES causes an increase in plasma E_2 levels of treated maternal rats and a decrease in plasma E_2 levels of 30-day-old rats. Finally, DES has no effect on the estrogen level of the ovarian homogenates of the maternal, fetal and 30-day-old young Long-Evans female rats.

ACKNOWLEDGEMENTS

I wish to express my profound gratitude to Dr. Roy Hunter, Jr. without whose tremendous help this work would never have been completed. Special thanks goes to Dr. John Mayfield for his help and advice throughout the preparation of this thesis, and also to Drs. Benjamin Shepherd and George Gass of Southern Illinois University (Carbondale, Illinois) for their critical evaluation of my thesis. Last but not the least, my thanks goes to my fiance', Joan Graham, for the wonderful support and encouragement she gave me throughout this period.

DEDICATION

" In Memoriam"

To my late father Rev. David 'Tade Okediji

and

My beloved late brother Prof. G. Oluokun Okediji

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CHAPTER I

INTRODUCTION

It is a well known fact that the incidence of cancer is on the rise. The incidence of cancer of the female genital organ is high. Conservative estimates place the total at more than 20% of all cancer. The uterus and mammary glands seem especially vulnerable. Normally, these organs are subject to spurts of growth which prepare them for function, after which they undergo retrogression. This generalization applies to both non-pregnant animals during estrous or menstrual cycles and to pregnant and lactating animals, the times of specific phases varying greatly in different conditions and species. These phases of growth, function, and retrogression are regulated by floods and ebb tides of ovarian and associated hormones (Allen, 1938).

Experimental production of genital and breast carcinomas in rodents, and rarely in monkeys, by means of estrogens has raised very important questions as to whether there might be some danger of inducing carcinomas in human beings by therapeutic administration of estrogens. Vaginal adenocarcinoma in young women was recently reported by Herbst, et al. (1971) as being associated with stilbestrol therapy of the mother during pregnancy. Recently, McClure and Graham (1973) described the occurrence of malignant uterine tumors in seven of ten squirrel monkeys following treatment with diethylstilbestrol. Herbst, et al. (1971) first noticed the correlation between adenocarcinoma of the vagina in adolescent girls following maternal ingestion of diethylstilbestrol. Greenwald, et al. (1971) confirmed the

same report in five cases that they studied which showed vaginal cancer after maternal treatment with synthetic estrogen. These problems have caused some justified and some unjustified concern over the use of estrogens post-ovulatory to prevent implantation.

Structural and functional abnormalities in the sex organs of male offspring of humans treated with diethylstilbestrol (DES) have been reported by Gill, et al. (1976). Among the abnormalities are epididymal cysts, hypotropic testes, and capsular induration of the testes. The National Institute of Child Health and Human Development (NICHD) reported that genital and sperm abnormalities were found in some of a group of men whose mothers received DES in an experiment when they were pregnant 22 years ago. They also reported in the study of Dr. Maluce Bibho (42 males who were exposed to DES) two cases of undersized testes, four cysts in the seminal duct and one testicular mass that might have been caused by injury. None of these abnormalities was seen in 37 men examined who were not exposed to DES. In addition, 29% of the men studied had severe semen abnormalities that could impair fertility; similar problems were not observed in the control group. However, it was noted that none of the abnormalities seen in the men was cancerous.

Current controversy rages around whether DES should be permitted as as after-the-fact contraceptive, the so-called "morning after" pill. Since it is almost certain that DES causes vaginal cancer, it should be noted that the important feature is in the timing of the exposure rather than the drug itself. During the crucial embryonic period, most cells are

preparing for their varied roles. At this time almost any unusual changes in their environment might set them up for disordered growth, perhaps cancer in later years. Most importantly, there is no good evidence that DES causes cancer in individuals exposed as adults.

Hormonal influence on tumor growth has been postulated to occur, and the occurrence has been evaluated by various mechanisms. Williams, et al. (1974) showed that the carcinogen ethylmethane sulphonate (EMS) induced the development of mammary gland tumors in female Sprague-Dawley rats. Treatment with EMS, however, failed to result in any significant added effect to tumor occurrence. Thus, they concluded that EMS induction of mammary gland tumors in this strain of rats was not potentiated by estrogens.

Estrogen has been implicated by its action with the alteration in biosynthesis of RNA, based on derived biochemical evidence that describes acceleration of DNA synthesis as a result of estrogen treatment, and from observations that inhibition of DNA-dependent RNA biosynthesis by treatment with actinomycin D prevents target organs from responding to the hormone. Segal, et al. (1965) added that biologically active RNA can be extracted from estrogen-treated uterine endometrium to the stimulated state. They concluded that after the hormone initiates RNA biosynthesis it is not necessarily involved in subsequent steps leading to the secondary morphologic changes in the uterus. With the multiplicity of problems related to therapeutic use of the synthetic estrogen and the lack of quantitative data on the problems of estrogen and cancer formation, this proposed study will advance the data on the effects of diethylstilbestrol (DES) on maternal

and the young female rat estrous cycle and any changes if any in their estrogen titer.

CHAPTER II

REVIEW OF LITERATURE

Studies on ovarian hormones in relation to female genital cancer were carried out about 40 years ago. Allen (1938) reported that ovarian hormones were obviously not the only cause of female genital cancer and that estrogen was a very important growth stimulator of female genital tissues. Acting in high concentration and continuously, these hormones produced atypical growth and were a determining factor in mammary and uterine cancer. Gardner (1939) reviewed the roles of estrogen in carcinogenesis and indicated that few investigators have openly committed themselves to the question, are estrogens carcinogenic? While another group of workers included estrogen in their discussions of chemical compounds as carcinogenic agents, he pointed out that hereditary factors were of great importance in determining the influence of estrogens on mammary cancer. Estrogen plays a significant role, however, in determining responses to the generally recognized carcinogenic chemicals. From the practical point of view, one must accept the conclusion that without the administration of exogenous estrogen, there were no mammary and few uterine tumors in female mice. In animals receiving chronic injections of comparatively high levels such tumors appear. It seems important that the question of the carcinogenic effects of estrogens is of great significance because of the therapeutic applications of these chemicals (Cramer, 1937).

Diethylstilbestrol (DES) is one of the synthetic estrogens available today for therapeutic use. It is a crystalline estrogenic substance

capable of producing all the pharmacologic and therapeutic responses attributed to natural estrogens. Dodds, et al. (1938) synthesized the non-steroidal estrogenic substances diethylstilbestrol. Besides being a potent estrogenic substance, this drug was equally effective when given orally and parenterally. Noller (1976) reported the various implications of DES on pregnancy and the lower reproductive tract changes, namely, changes in the epithelial lining of the cervix and vagina in offspring of mothers treated with DES. Thus, DES has been used for the treatment of menopausal syndrome, senile vaginitis, Kraurosis valvae, and breast cancer (Smith, 1965; Hill, 1972; Fetherston, 1975; Wharton, 1975). Several side effects have been reported between maternal ingestion of DES during pregnancy or as a post-ovulatory agent to prevent implantation and a possible occurrence of carcinoma in women known to be aborters (Bongiovanni and McFadden, 1960). Smith (1956) reported the use of diethylstilbestrol in the prevention and treatment of pregnance complications.

To date, there have been few, if any reports on the effects of DES treatment on the estrous cycle of maternal or neonatal rats. A lot of variablility occurred in describing the stages of the rat's estrous cycle. Long and Evans (1922) described cytological changes in the vaginal smear of rats between the epithelial stage of proestrous and the complete leucocyte infiltration of dioestrous. Staples and Geils (1965) further substantiated in a more detailed manner the cornified cells seen after the period of "Cell paucity" in diestrous, 12 hours before initiation of proestrous. They reported that shortly after this period, the smear consisted

of keratinized and large angular-appearing prekeratinized cells with some large basophilic epithelial cells in addition to a few leucocytes.

Ovarian tumors in mice have been induced chemically. Merchang (1958) induced granulosa-cell tumors of ovary in mice of various strains by fortmightly painting them with a solution of 0.5 percent 9:40 dimethyl-1:2 benanthracene (DMB) in olive oil. Grean (1941) reported the susceptibility of uterine adenomata as a function of constitutional factors. He concluded that the association of two disorders occurred as a result of liver damage, incident to toxemia, which impaired the function of this organ in relation to estrin inactivation. Also the concentration of this substance in the blood stream subsequently rose to a carcinogenic level.

A consideration of the disparity in the distribution of uterine growths in rabbits and women indicated that the absence of squamous epithelium in the cervix of the rabbit and the physiological activity of the endometrium of the fundus in this species were the determining factors. Recently Williams, et al. (1974) and Vorhees (1973) studied the influence of hormone on rats treated with the carcinogen ethyl methanesulphonate (EMS) which caused development of mammary gland tumors in female Sprague-Dawley rats. They indicated that hormonal therapy induced and/or enhanced tumor production in certain animal models. Similar findings were reported by Bertram and Craig (1972) and Kirkman (1972). DES administration along with the carcinogen EMS failed to cause an increased production of tumors in their study. A lowered incidence of mammary tumors occurred to a lesser extent in the presence of hormonal therapy.

Estradiol is the most biologically active estrogen produced by the gonads in humans (Emment, et al., 1972). The immediate metabolite, estrone, serves as the principal precusor for the formation of the other urinary estrogens. The natural sources of estrogen in humans are the gonads, adrenals and placenta. In males and prepubertal females only minimal amounts of estrogen are excreted. With the establishment of the mentrual cycle, the daily estrogen levels show a biphasic pattern of excretion, with low levels at the menstruation period (Brown, 1955). In rats, for example, the estrogen levels vary greatly with the phases of the estrous cycle. Roy and Brown (1960) developed a chemical method for the estimation of estriol, estrone and estradiol-17B in the blood of pregnant women and of the fetus, based on a micro-modification of the Kober Colour reaction: A method suitable for day-to-day routine use, four determinations being possible in 1½ working days. This method was a modification of that previously reported by Veldhuis (1953) and Preedy and Aitken (1957) where estrogens were measured fluorimetrically by the fluorescence which develops when they are heated with sulphuric acid. Many of the impurities in the estrogen fractions interfered in the measurement, either by fluorescing themselves or by partially quenching the fluorescence of the estrogen. Veldhuis (1953) introduced corrections for this interference and obtained more acceptable figures, but many assumptions were involved so that proof or reliability of the results was difficult. Preedy and Aitken (1957) derived the specificity of their results from following the behavior of the flourescent materials during fractional gradient elution chromatography.

This experiment involved fluorimetric analysis of large numbers of fractions in the chromatograms, and though it gave excellent results, it was not suited to the assay of large numbers of blood samples.

Recently, the advent of radioimmunoassay for steriod and protein hormones (Wu and Lundy, 1971; Emment, et al., 1972) has made it possible to measure these hormones in small samples of blood from individual animals. In order to efficiently utilize these blood samples for steroid measurement, Shaikh (1971) developed a comprehensive method that would enable the different steroids to be measured by one extraction procedure. For the estrogens, phenolic partition (Baird, 1968), followed by celite microcolumn chromatography (Abraham, et al., 1970), was found useful.

With the multiplicity of problems relating hormone to cancer and various other abnormalities, it is still highly speculative to attribute the formation of carcinoma in progenies solely to the idea that the mothers were at one time or the other exposed to synthetic estrogen (DES). Several factors unknown in the epidemiology of DES relation to later vaginal carcinoma are still present. Is there an enzymatic reation of the DES with certain cells during embryogenesis for this effect to be induced (Connolly, et al., 1973)? Is the effect dose related, or is the duration of the therapy more critical than the timing of the therapy in producing the observed carcinoma (Hill, 1972; Herbst, et al., 1975)? Lastly, is it possible that DES alters maternal or fetal vaginal cells in the uterus with changes that do not become manifested in a malignant form until later years (Mori, 1969)? Furthermore, Herbst(1971, 1975) reports suggested in human cases

they reviewed whose progenies exhibited carcinoma and prior to received progesterone to prevent abortion, yet not all in the reviewed cases received DES.

CHAPTER III

MATERIALS AND METHODS

Adult Long-Evans female rats weighing 150-200 g and 30-day old young female Long-Evans rats withing between 70-100 g of the strain LE:BLU were used in these experiments. They were obtained from Blue Spruce Farms, Altamont, New York. Both groups of rats were divided into three groups: (1) the untreated control, (2) the manipulative control treated with saline (2 cc/kg body weight), and (3) DES-treated group (administered with diethylstilbestrol, 35 mg/2 cc/kg of diethylstilbestrol-DES dissolved in deionized water at room temperature, without exposure to light via stomach intubation. The DES was obtained from Sigma Chemical Co., St. Louis, MO. The manipulative controls were administered saline in the same manner, via stomach intubation. All of the rats were maintained at a 12 hr light and 12 hr day/night cycle.

The estrous cycle of the rats in these studies was monitored for three weeks by taking daily vaginal smears in the a.m. to determine whether they showed a 4 or 5 day cycle. The rats were fed the standard laboratory rat chow, and water was given ad libitum. At the end of the monitoring periods, those rats showing 4 or 5 day estrous cycles were utilized in the study. They were then treated with the above mentioned dosage of DES and saline once. Daily vaginal smears were collected in the a.m. to determine any changes in the cell types, and the estrous cycle stage of each rat for about 4 weeks.

The daily vaginal smears were stained with methylene blue and observed

under the light microscope to determine the type of cells present and the stage of the cycle. Vaginal smear cell count was determined in 0.5 cc saline smear volume stained with methylene blue and counted under the light microscope. It was determined that no more than 300 cells per smear volume per slide can be counted for each stage of the estrous cycle of the three groups of adult rats. Only one slide was counted for each of the young adult rats in the control, saline control and the DES-treated group regardless of the stage of estrous cycle, and after treatment effect commences.

Plasma Collection

Maternal: Blood was collected in heparinized tubes after anaesthesia. The blood samples were then centrifuged at 1500 g for 10 min and the plasmas were then frozen until assayed.

30-day old rats: The rats were decapitated at duration of the experiment and the blood collected in heparinized tubes. The plasma samples were collected as described above.

Ovary Collection

Maternal: At the end of a 4 week observation period after treatment, the female rats were mated and the day that each was sperm positive designated ½ day. After 19½ day gestation period, the rats were etherized, the fetuses, the ovaries (collected on ice) were removed. The collected ovaries were homogenized in 10 cc of phosphate buffer with Sonifer Cell Disruptor (Model W-140D, Ultrasonics, Inc., Plainview, New York). The homogenates were then kept frozen until assayed.

30-day old female rats: At duration of the observation period after treatment, the rats were decapitated and the ovaries removed and homogenized as described.

Radioimmunoassay of Ovarian and Plasma Estrogen

The procedure used to measure the estrogen level in the plasma and ovarian homogenate was similar to that described by the New England Nuclear Biochemical Co. and that described by Verma, et al. (1976). The estradiol/Estrone radioactive kits were purchased from the New England Nuclear Biochemical Company. Estradiol 17B (E_2) antiserum used was obtained from Dr. Edward Davis of the National Institutes of Health. Briefly, the procedure used was as follows:

Sample preparation: One ml sample was pipetted into a 13 ml glass stoppered centrifuge tube plus 1000 cpm of ^{3}H-Estradiol and then vortexed. The tubes were then incubated for 15 min at room temperature. Five ml of fresh ether was added to each tube and shaken vigorously for 60 sec. The phases were then left to separate. The ether phase was pipetted off and frozen in acetone-dry ice and collected into another vial. The ether extracts were taken to dryness under jet in a water bath at 37° C. One ml of solvent (methanol: Benzene: 9:1 v/v) was added to each vial and mixed thoroughly.

Separation: Each sample was separated in Sephadex LH-20 in glass columns 0.7 cm diameter, 15.0 cm long and 250.0 ml reservoirs, with a Teflon stopcock. The colums were washed continously with 20-30 ml solvent

prior to use.

Each sample was applied to the top of the column with 1 ml solvent thrice, and discarded. The samples were then eluted with 3.5 ml solvent which was collected as estrone, followed with 1 ml solvent which was discarded. Solvent (4 ml) was again added and collected as estradiol. The fractions were collected in vials and dried under a stream of air at 37° C.

Radioimmunoassay: One ml of benzene was added to each sample vial and vortexed, 100λ of each sample was taken into scintillation vials with 2 ml p-dioxane plus 8 ml of liquifluor and then thoroughly mixed. The samples were then counted for 2 min in LS-255 Beckman Liquid Scintillation Counter as recovery, which was about 54% for the assay.

For the assay sample, 0.9 ml of each E_2 of sample was transferred to assay tubes and dried completely under stream of air at 37° C in a water bath. The entire assay was run in aqueous medium (diluent) prepared by adding Bovine gamma globulin Fraction II, 1 mg/ml, to commercial normal saline (Abbott). All pipetting was done with glass lambda pipettes. The bound fraction was separated from free fraction by adding dextran coated charcoal, prepared by adding 100 mg Dextran T-70 to 200 mg Norit plus 100 ml of diluent. The solution was stirred with a magnetic stirrer at 4° C for 3 hrs prior to use in the cold. Estradiol standards were prepared by dilution techniques.

Procedure: Tubes were set up in duplicates for the zero or blank, standards and samples, i.e. 0, 10, 25, 50, 100, 250, 500, 750, 1000 pg of standard solutions were pipetted from each sample duplicate. For the total count and non-significant, 0.5 ml of diluent was pipetted. About

0.5 ml of tracer (10,000 cpm) was pipetted to each tube, followed with 0.5 ml of antiserum dilution that gave 50% binding to each tube, except the total count and non-significant tubes. Each tube was vortexed and incubated at room temperature for 3 hrs or overnight at 4° C. Exactly 0.4 ml of charcoal solution was added to each tube in the cold (4° C) with a micropipette. Again the tubes were vortexed and kept at 4° C for 30 min or overnight. The tubes were then centrifuged in a refrigerated centrifuge (Sorvall) at 2500 g at 4°C for 20 min. The supernatant from each tube was decanted into counting vials with 2 ml of p-dioxane and 8 ml of liquifluor, thoroughly mixed and counted for 2 min.

CHAPTER IV

EXPERIMENTAL RESULTS

During the synchronization period, all the rats utilized in the study exhibited either a 4 or 5 day cycle, undergoing the four stages of the estrous cycle, namely, proestrous, estrous, metestrous, and diestrous.

Vaginal Smear of the Treated and Untreated Adult Female Rats

The control (untreated rats) maintained a synchronous 5 day cycle, exhibiting all four stages of the estrous cycle as described in Fig. 1a-d. The saline treated manipulative control rats also exhibited a 5 day cycle, which occurred. The four stages of the estrous cycle (Fig. 2a-d) was maintained after treatment with 2 cc of saline via stomach intubation. All of the 35 mg DES treated group of rats showed a 4 day cycle before treatment (Fig. 3a-d). Five days after treatment two to the rats remained in persistent estrous for six and four days, respectively, but later returned to a normal 4 day cycle. The other rats stayed in early estrous stage for four days, three days after treatment; however, as in the former two rats, their cycle became synchronous thereafter.

Following 30 days of daily vaginal smear observations after DES treatment, all fifteen adult female rats were mated. The day each rat was sperm positive, that is, when tails of spermatozoa were observed among the cornified epithelial cells in the vaginal smears (Fig. 4), was designated ½ day of pregnance. All but three of the rats were pregnant at 19½ day gestation period. Two other rats were less than 19½ day gestation period at surgery.

Fig. 1a. Maternal rat estrous cycle for normal control. Proestrous stage typifying mainly nucleated epithelial cells arranged in rows.

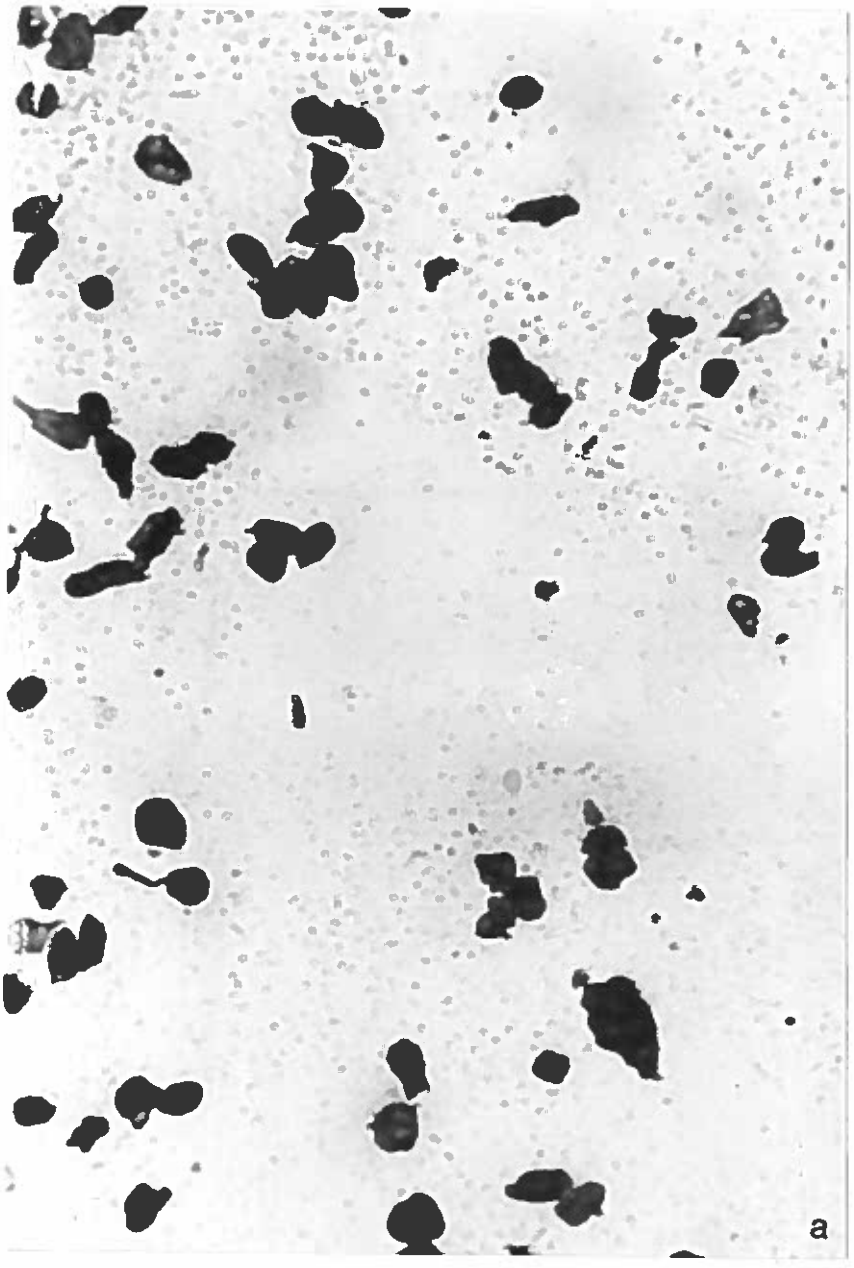

a

Fig. 1b. Maternal rat estrous cycle for normal control. Estrous stage with predominantly cornified epithelial cells.


b

Fig. 1c. Maternal rat estrous cycle for normal control. Metestrous stage showing white blood cells and cornified epithelial cells.

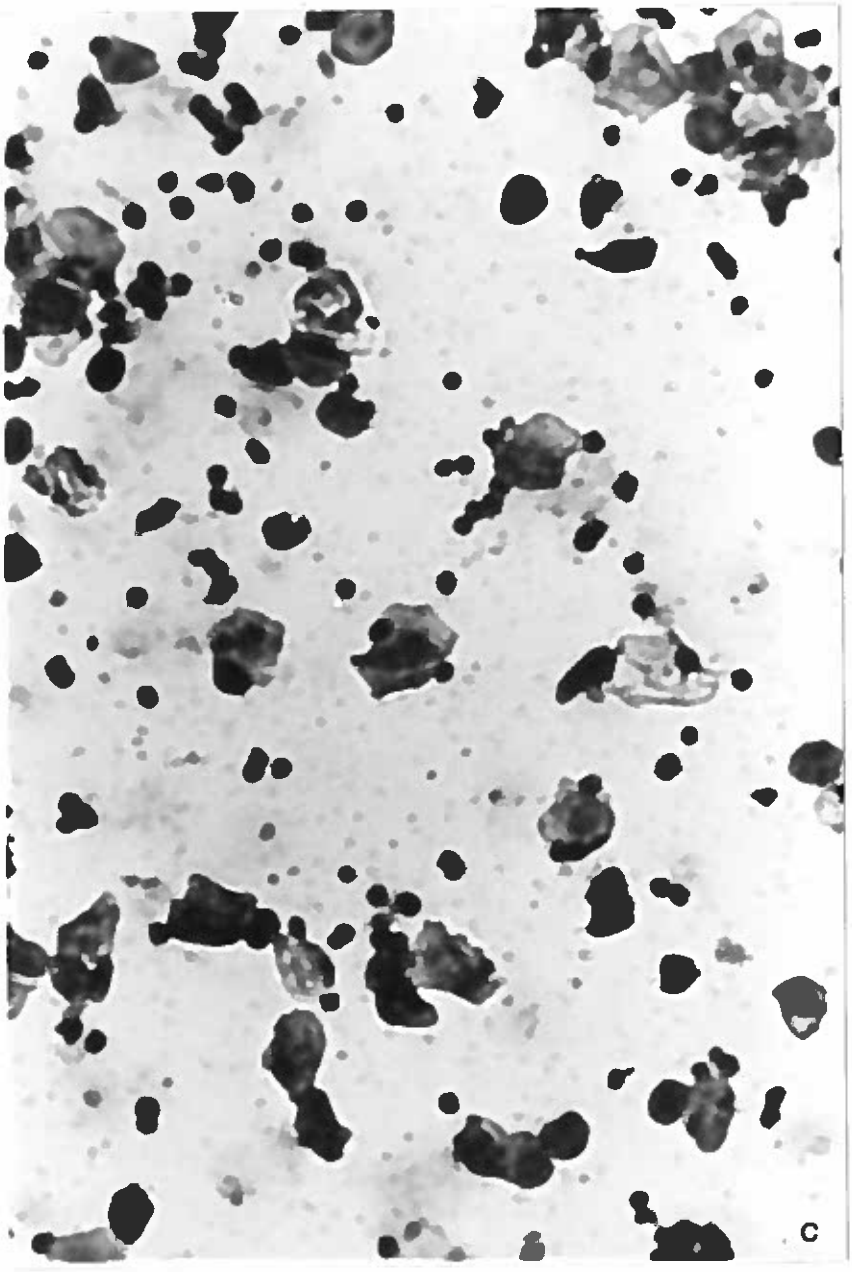

C

Fig. 1d. Maternal rat estrous cycle for normal control. Diestrous stage showing predominantly leucocytes (white blood cells).


d

Fig. 2a. Maternal rat estrous cycle for saline control. Proestrous stage showing predominantly nucleated epithelial cells arranged in rows or clusters.

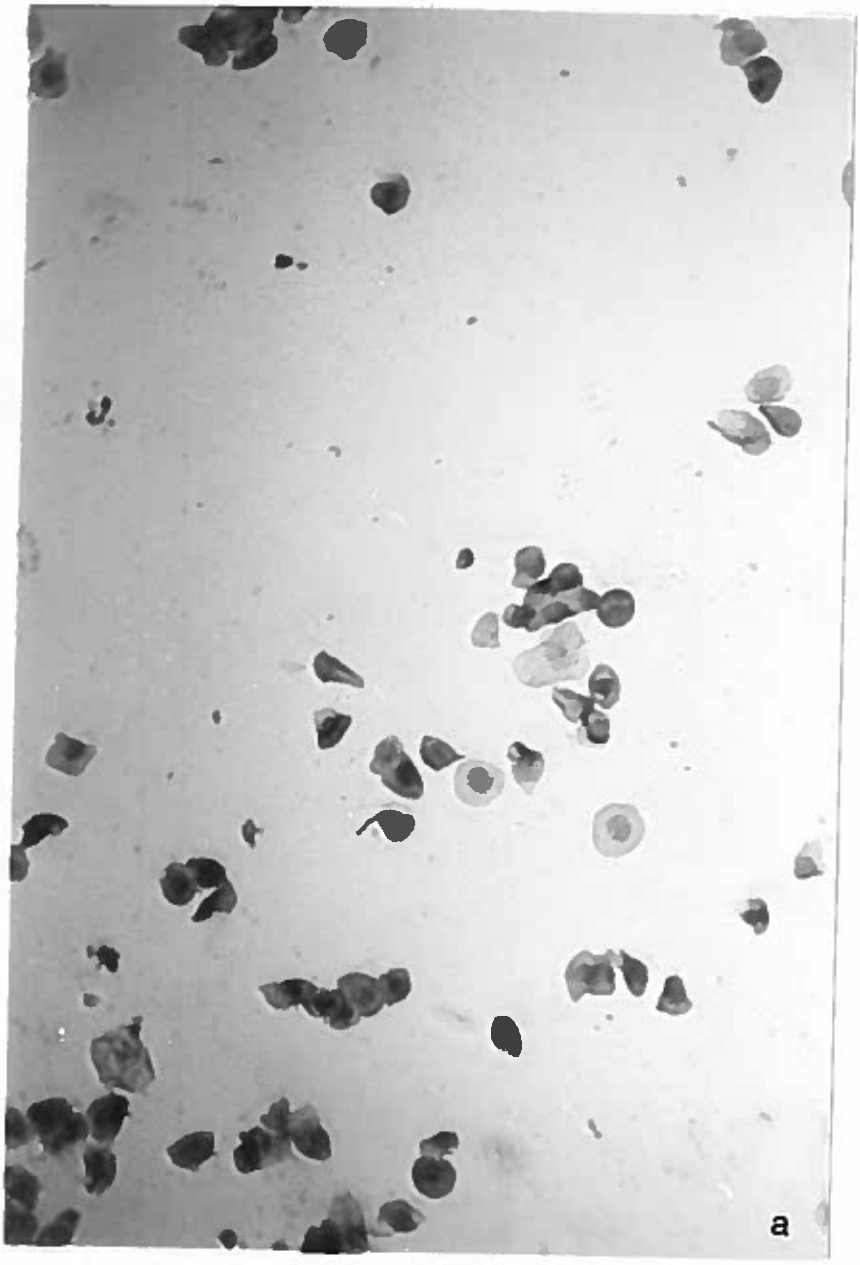

a

Fig. 2b. Maternal rat estrous cycle for saline control. Estrous stage showing mainly cornified epithelial cells.

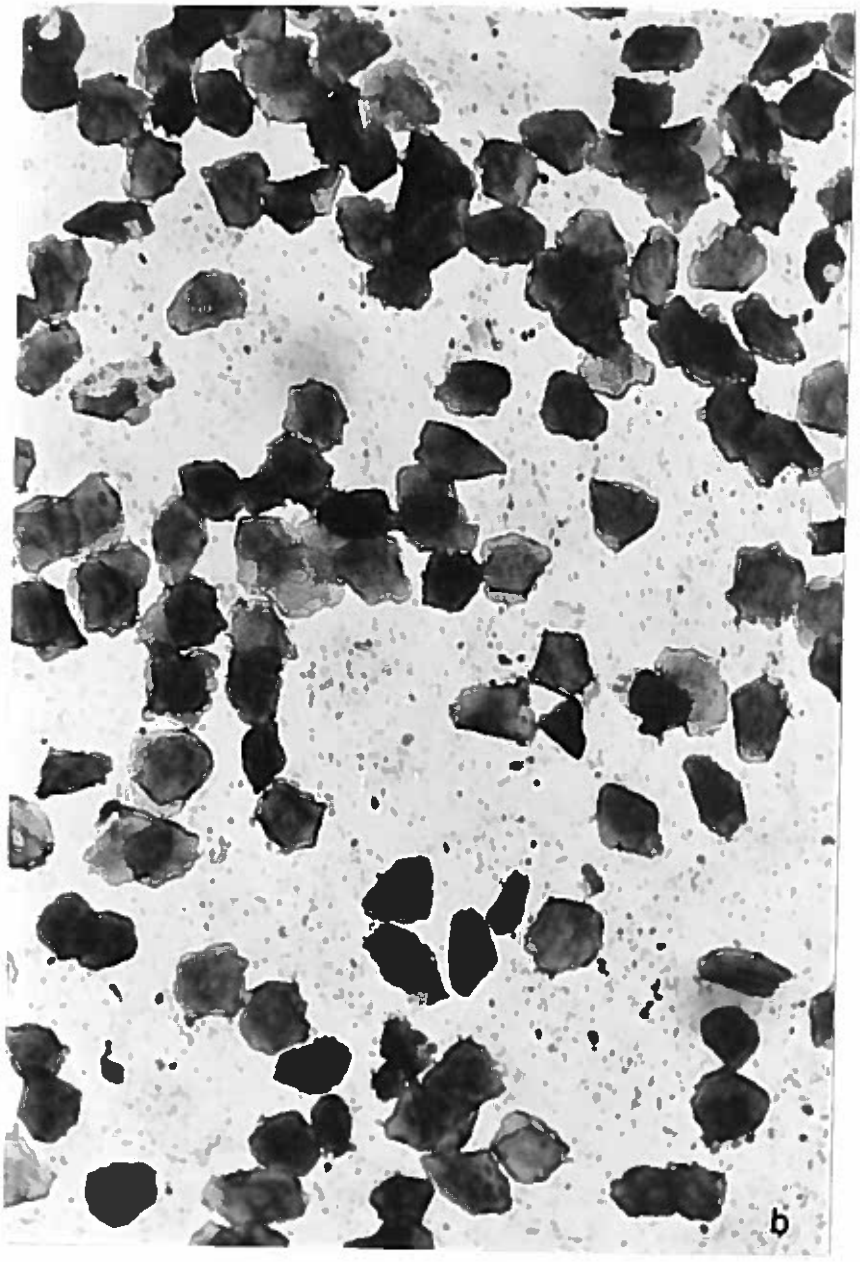

b

Fig. 2c. Maternal rat estrous cycle for saline control. Metestrous showing mostly white blood cells and cornified epithelial cells.

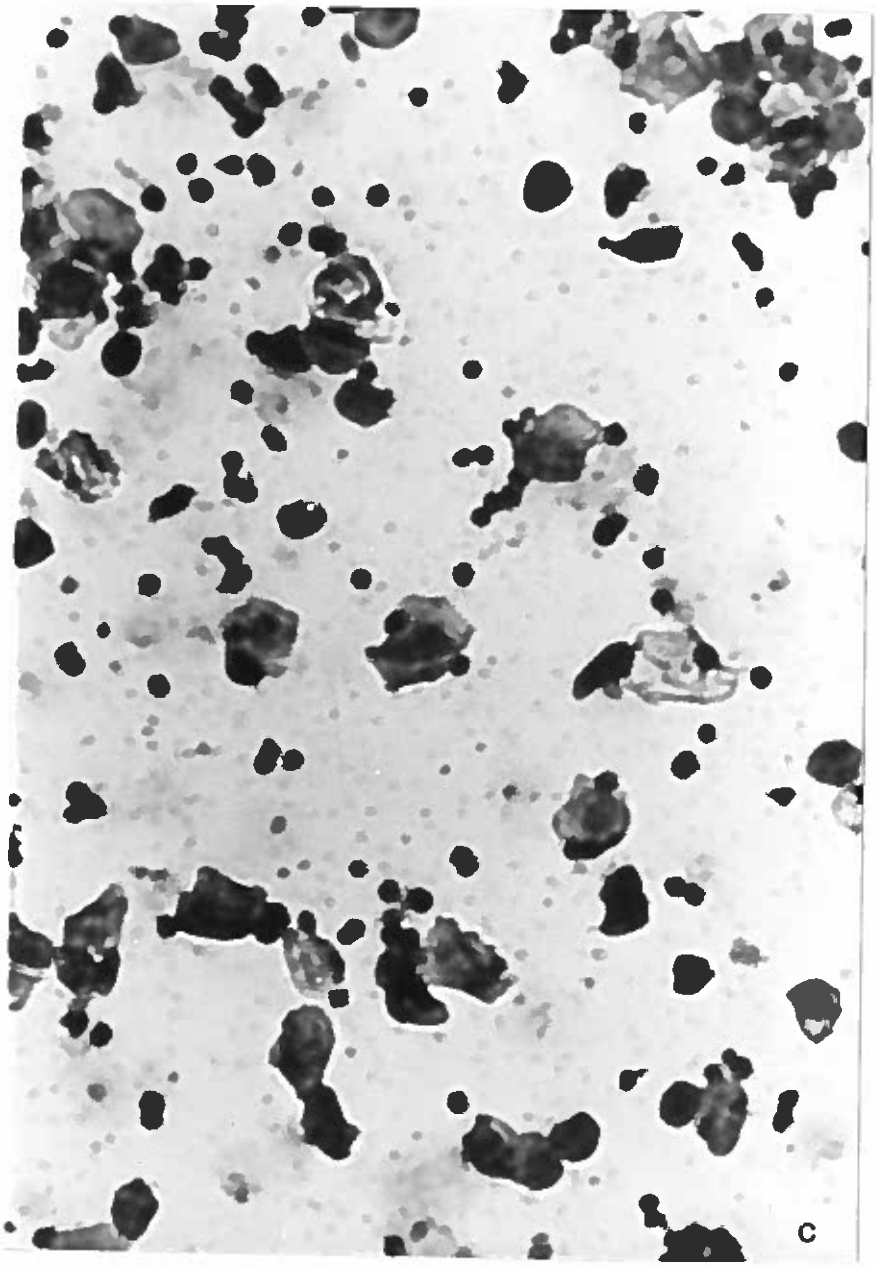

C

Fig. 2d. Maternal rat estrous cycle for saline control. Diestrous stage showing white blood cells predominantly.


d

Fig. 3a. Maternal rat estrous cycle for DES treated group. Proestrous stage showing mainly nucleated epithelial cells.

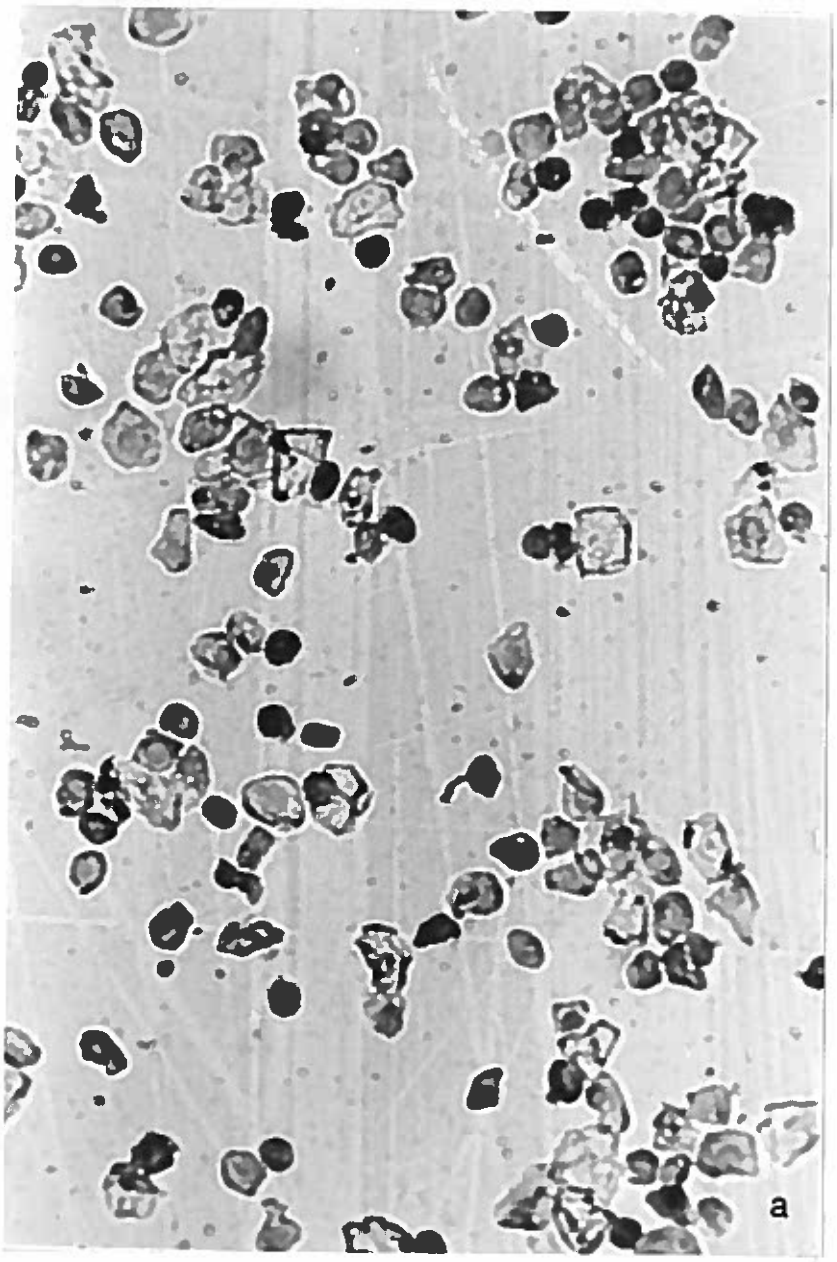

a

Fig. 3b. Maternal rat estrous cycle for DES treated group. Estrous stage showing clusters of cornified epithelial cells.


b

Fig. 3c. Maternal rat estrous cycle for DES treated group. Metestrous stage showing few cornified epithelial cells and white blood cells.

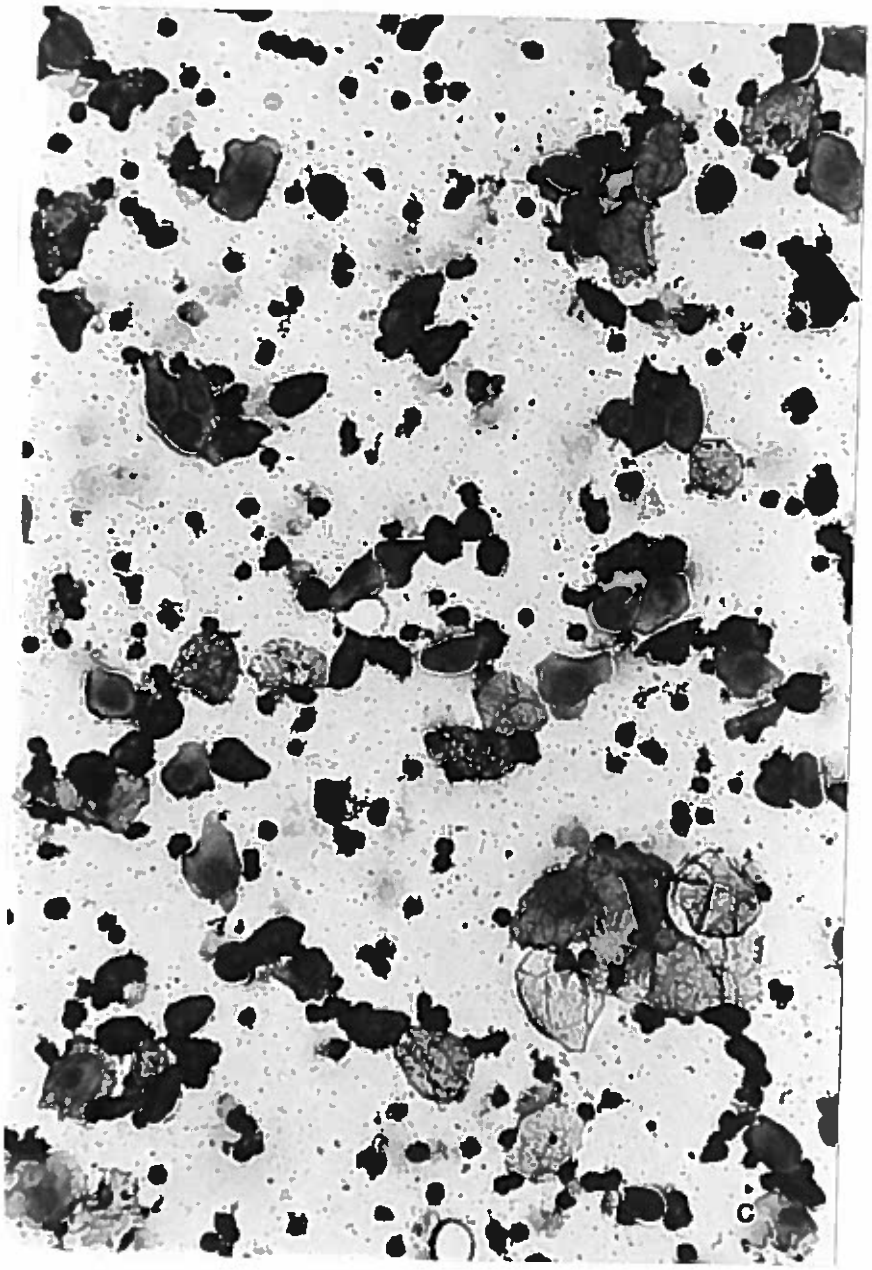

C

Fig. 3d. Maternal rat estrous cycle for DES treated group. Diestrous stage showing predominantly white cells (leucocytes).

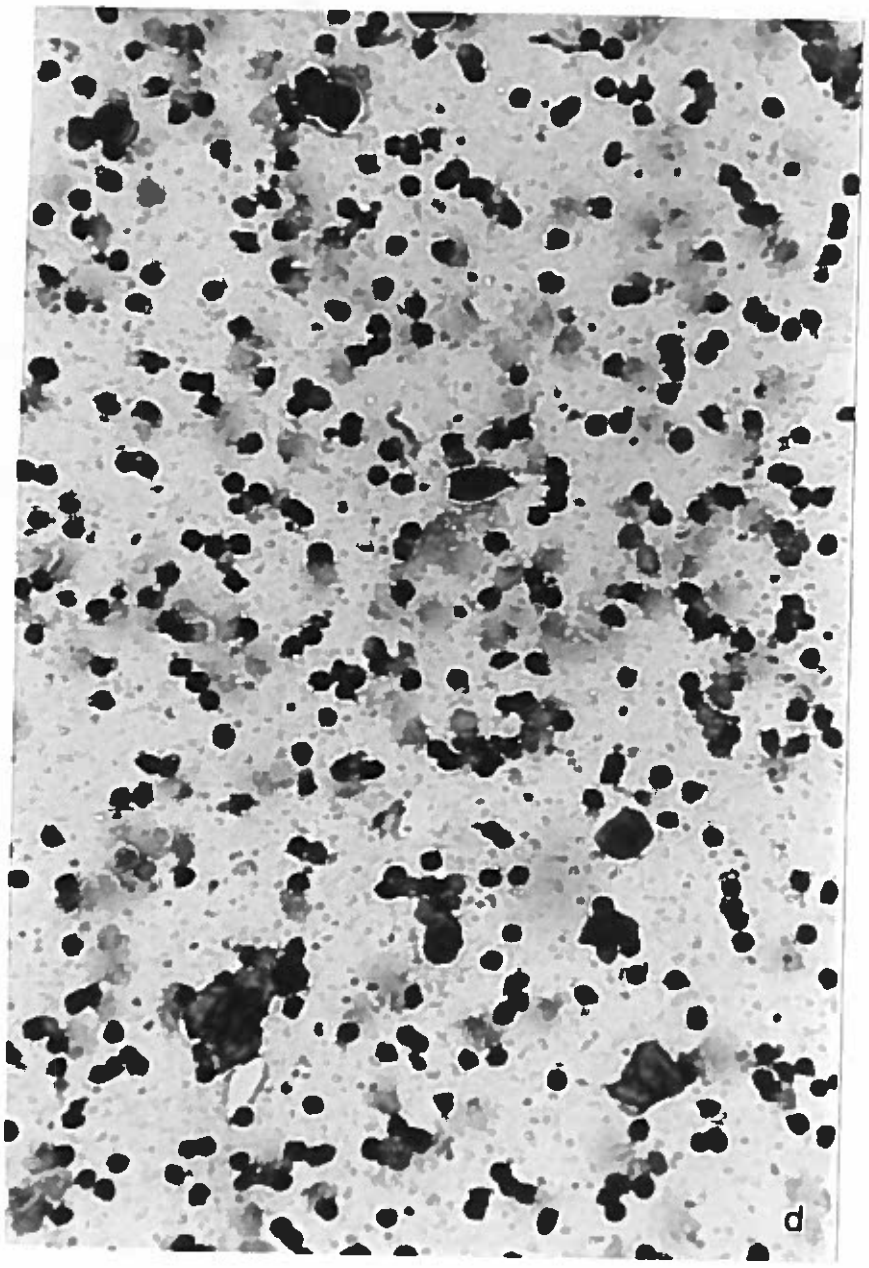

d

Fig. 4. Vaginal Smear of Sperm Positive Adult Female Rat. Note the sperm tails among the cornified epithelial cells.

Vaginal Smears of the Treated and Untreated 30-Day Old Female Rats

The control rats maintained a synchronous 5 day cycle and showed all four stages of the estrous cycle (Fig. 5a-d). The saline treated manipulative control rats exhibited a 4 day cycle, undergoing all four stages of the estrous cycle (Fig. 6a-d). This group was maintained after treatment with 2 cc of saline, as described previously. One of the rats in this group did maintain an estrous stage for 3 days after treatment with saline, then later returned to the normal 4 day cycle. Of the fifteen 35 mg DES treated rats, all showed a 5 day cycle before treatment (Fig. 7a-d). Ten days after treatment, eight of the rats remained in persistent estrous for 25 days. On the 26th day the vaginal smear stage was metestrous, where a few white blood cells were present along with numerous cornified epithelial cells. Four other rats remained in persistent estrous 5 days after treatment. Twelve days later the same rats changed from estrous to metestrous. Thereafter, the smear stages alternated between 4 days of persistent estrous and two days of diestrous. Also, two other rats remained in persistent proestrous (Fig. 7a) 13 days after treatment for 6 days; thereafter, the vaginal smear cycle stage alternated between 3 days of persistent estrous and proestrous, respectively. The last rat remained in persistent estrous 13 days after treatment (Fig. 7b). Data for all rats have been summarized in Fig. 8.

In the vaginal smear cell count of the adult female rats, slides were prepared for each stage of the estrous cycle for each individual rat of

Fig. 5a. 30-day old female estrous cycle of normal control. Vaginal smear of rat at proestrous. The predominant cells are nucleated epithelia cells.

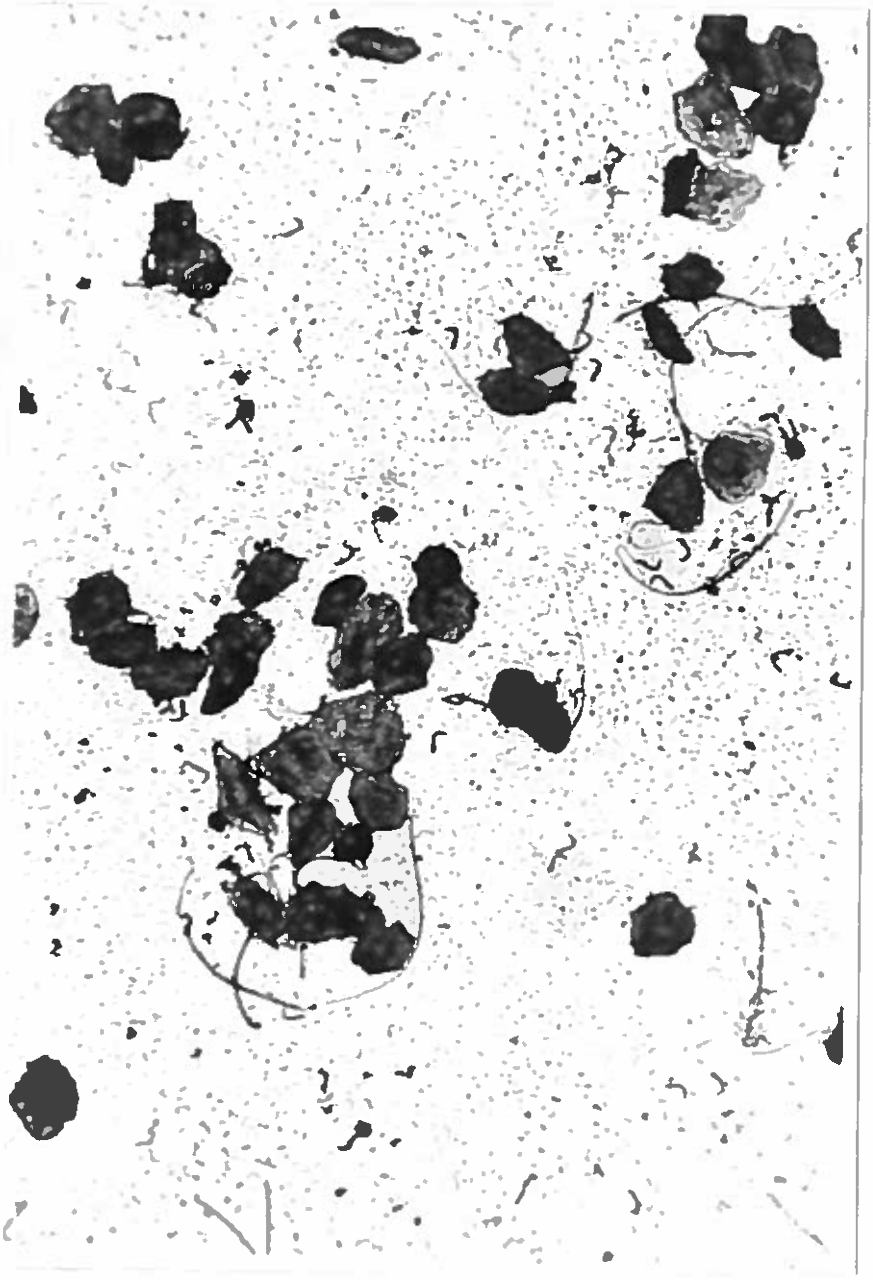


Fig. 5b. 30-day old female estrous cycle of normal control. Vaginal smear at estrous. Predominant cells are cornified epithelia cells.

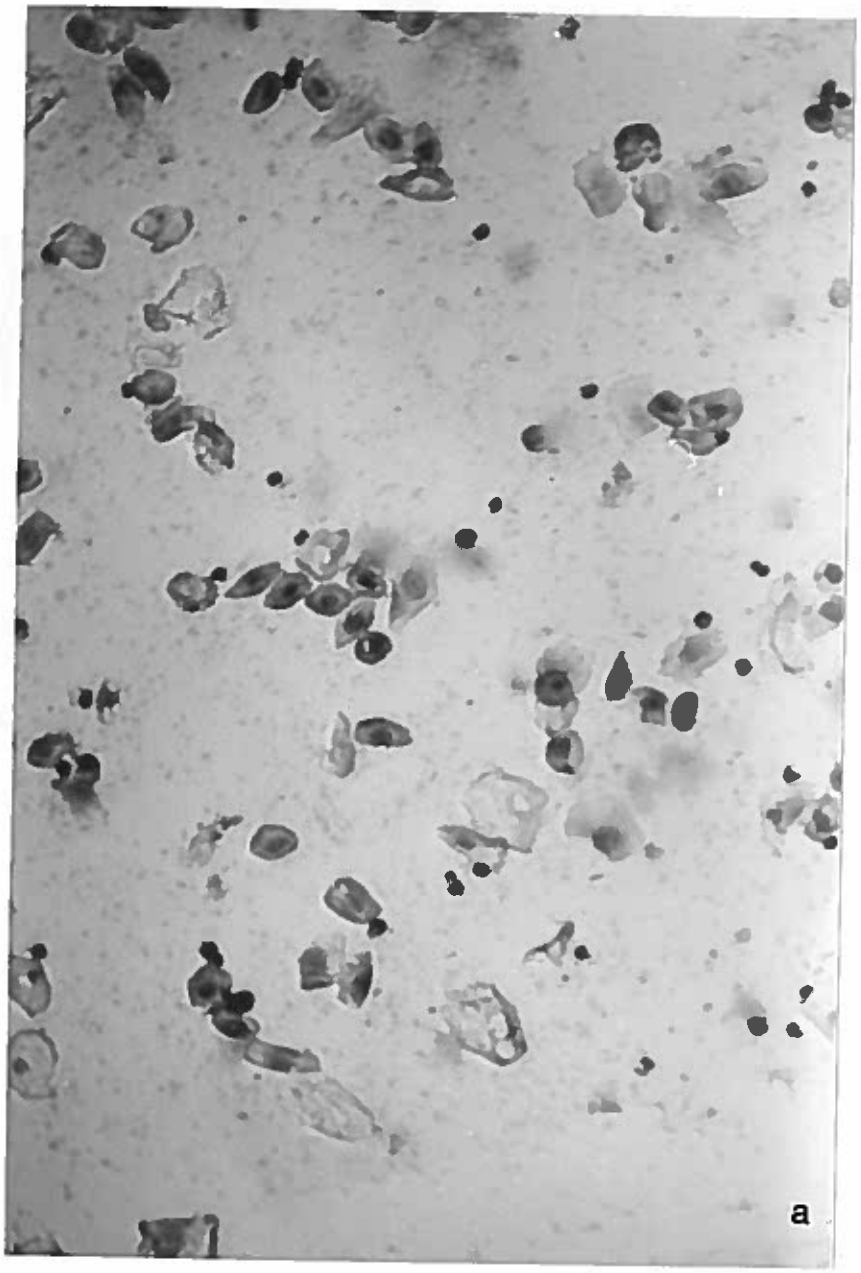

a

Fig. 5c. 30-day old female estrous cycle of normal control. Vaginal smear at metestrous. Mostly the cells are of the cornified epithelial cells with few leucocytes.


b

Fig. 5d. 30-day old female estrous cycle of normal control. Vaginal smear at diestrous. White blood cells (leucocytes) predominate with scanty nucleated epithelial cells.


C

Fig. 6a. 30-day old rat estrous cycle for saline control. Proestrous smear showing rounded nucleated epithelial cells and very scanty cornified cells.

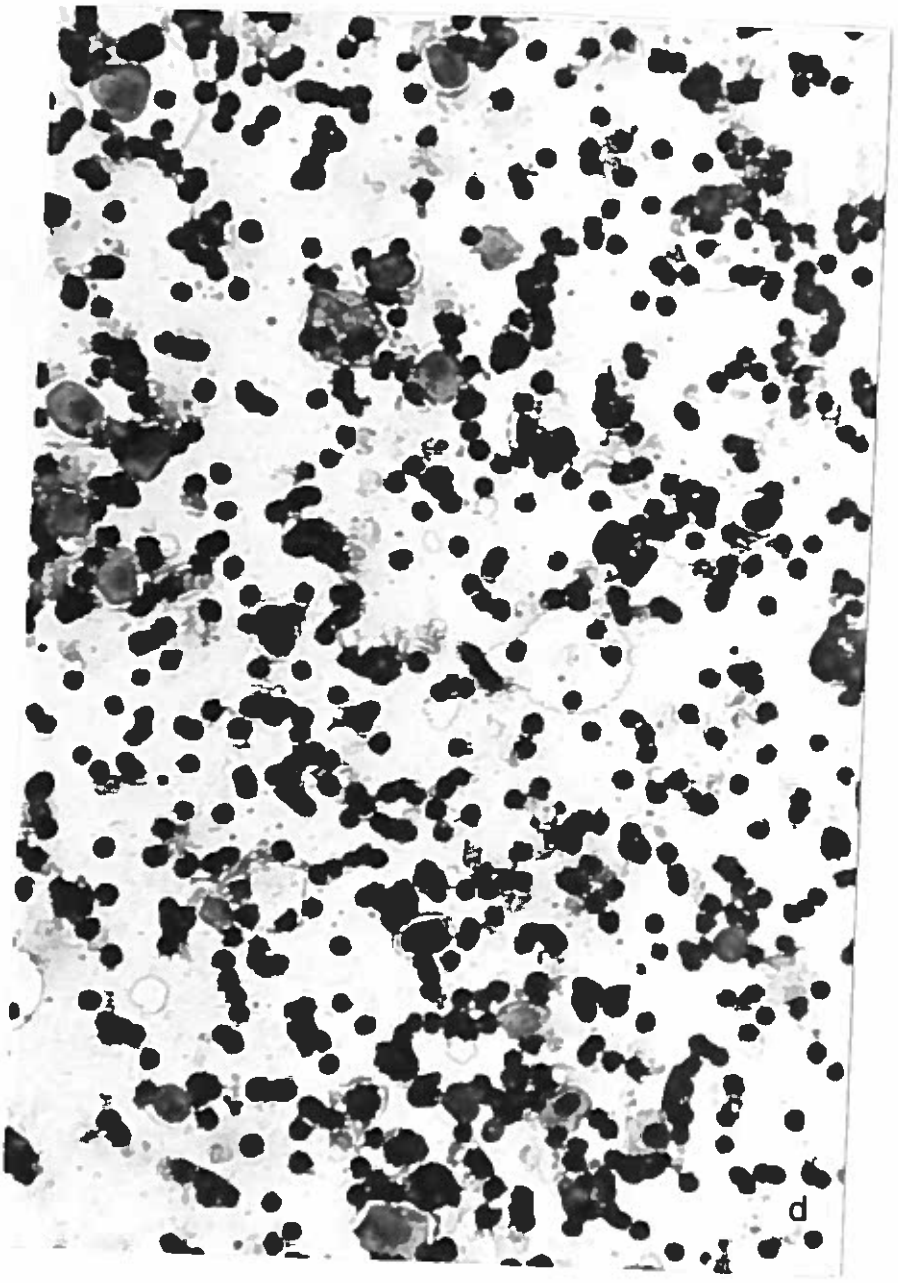

d

Fig. 6b. 30-day old rat estrous cycle for saline control. Estrous smears showing few leucocytes and cornified epithelial cells.

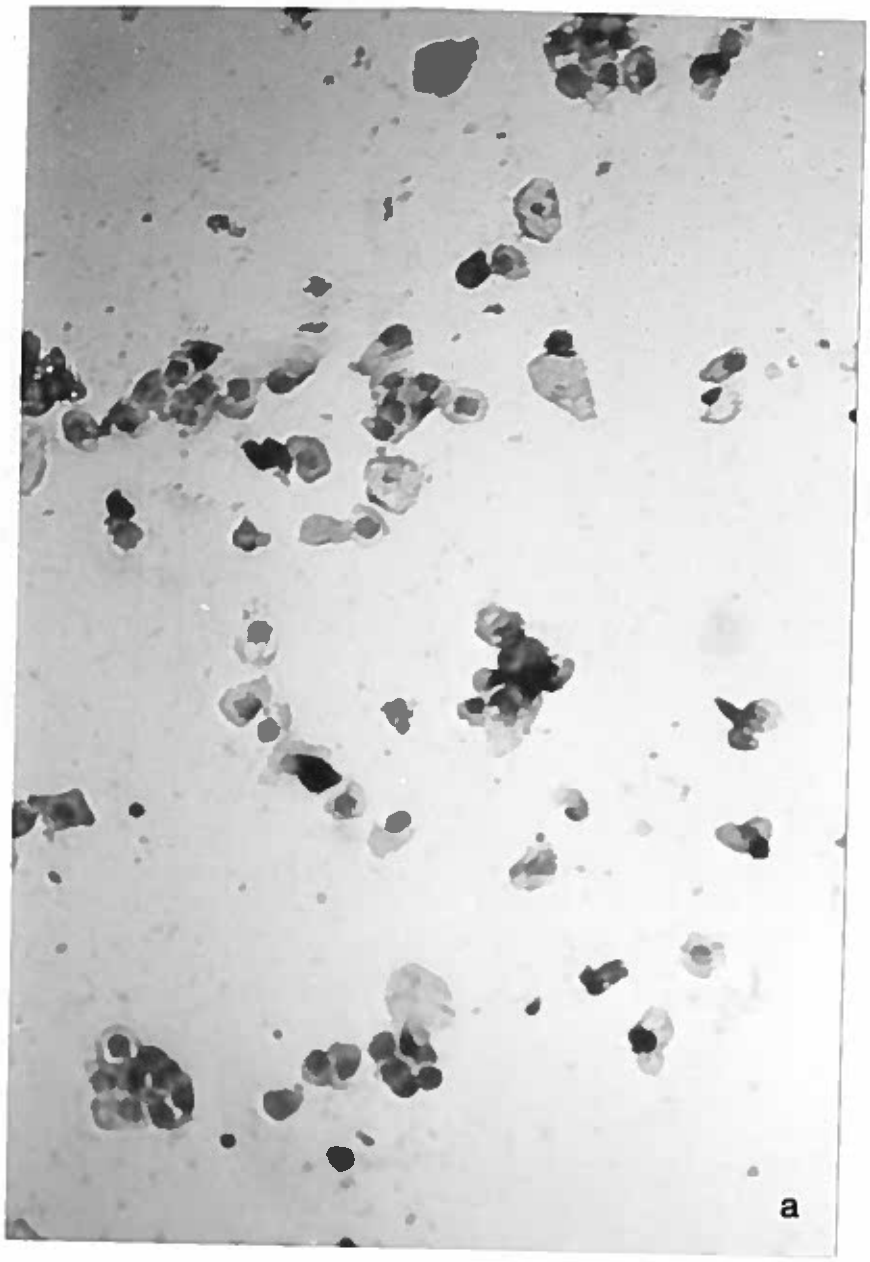

a

Fig. 6c. 30-day old rat estrous cycle for saline control. Metestrous smears showing few leucocytes and cornified epithelial cells.

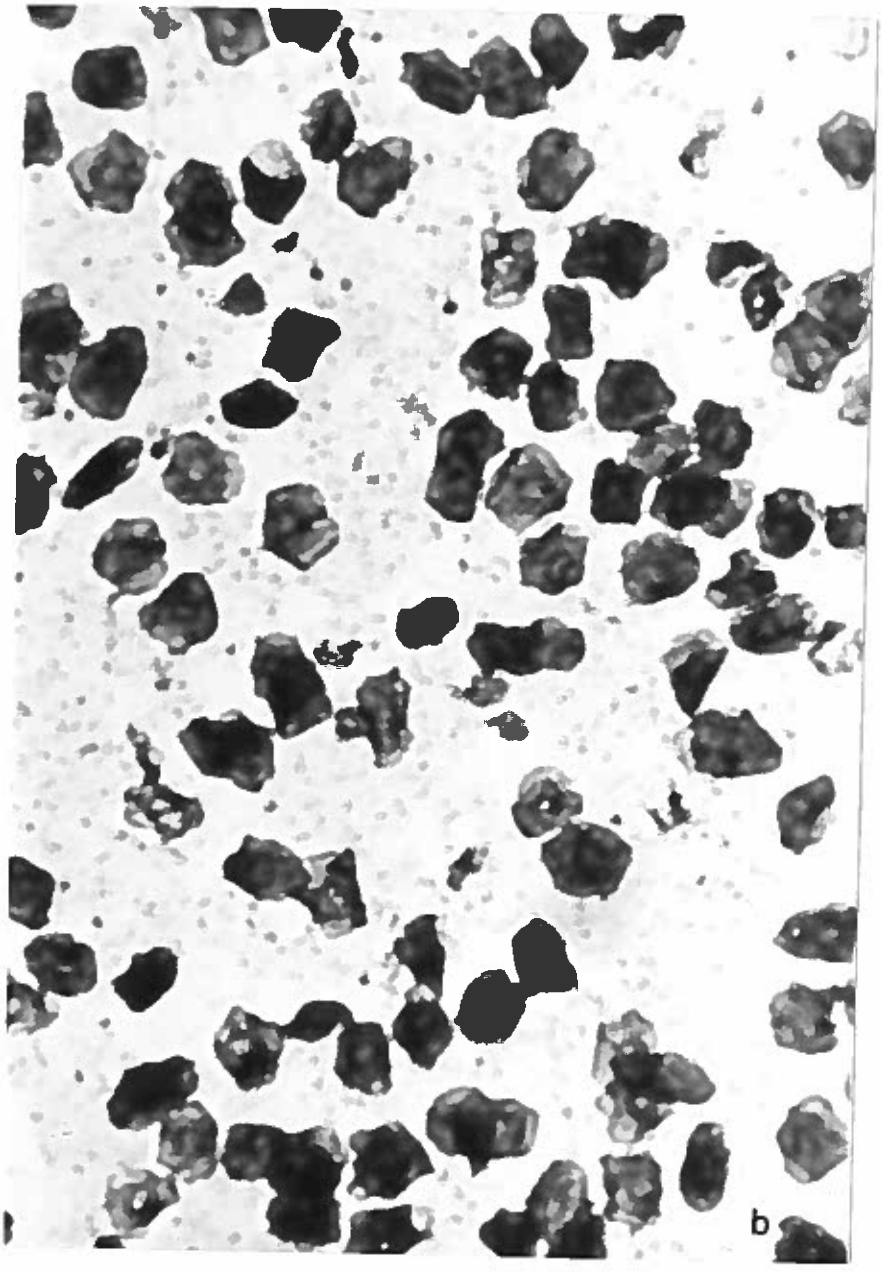

b

Fig. 6d. 30-day old rat estrous cycle for saline control. Diestrous smear showing predominantly leucocyte cells and a few epithelial cells.

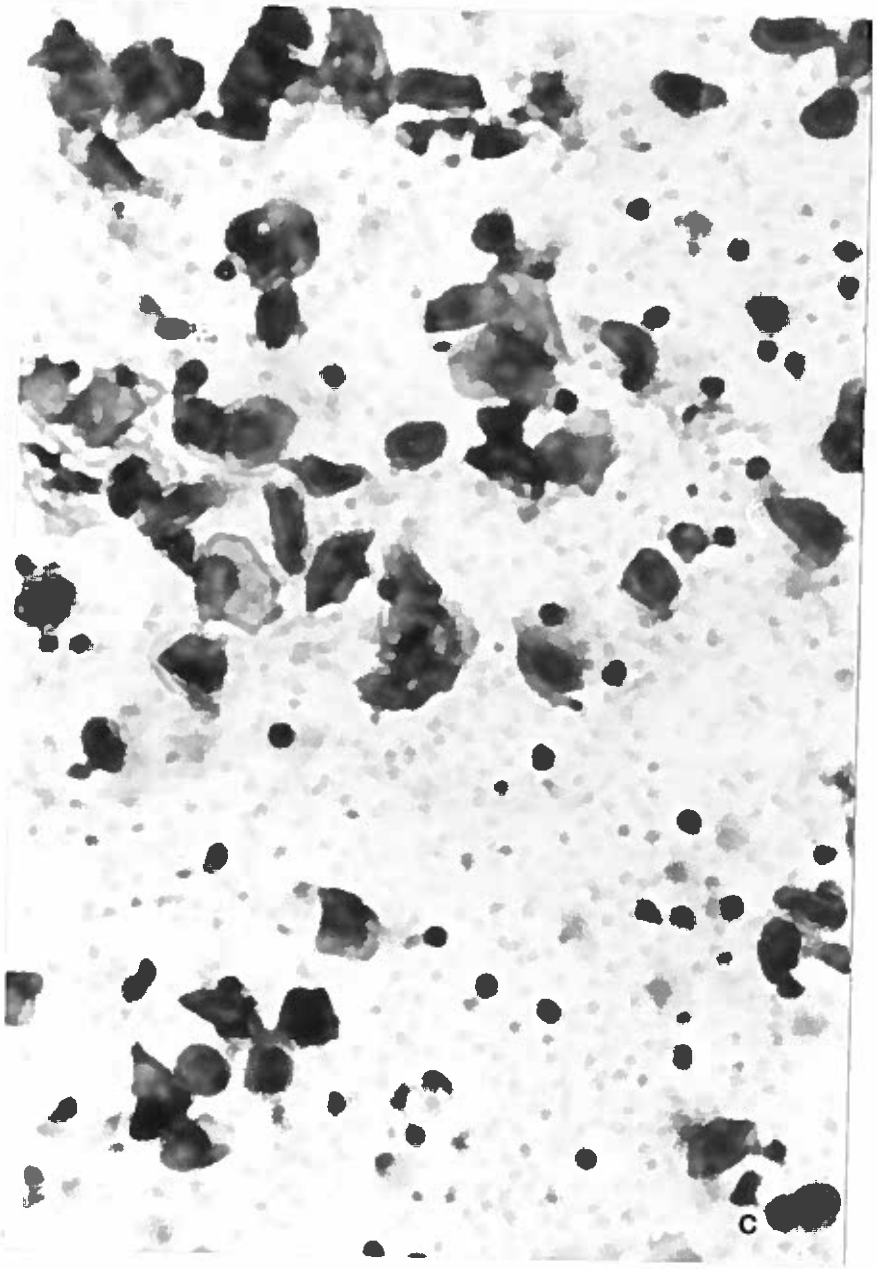

C

Fig. 7a. 30-day old rat estrous cycle for DES treated group. Pro-estrous smear showing rounded nucleated epithelial cells with few cornified cells.

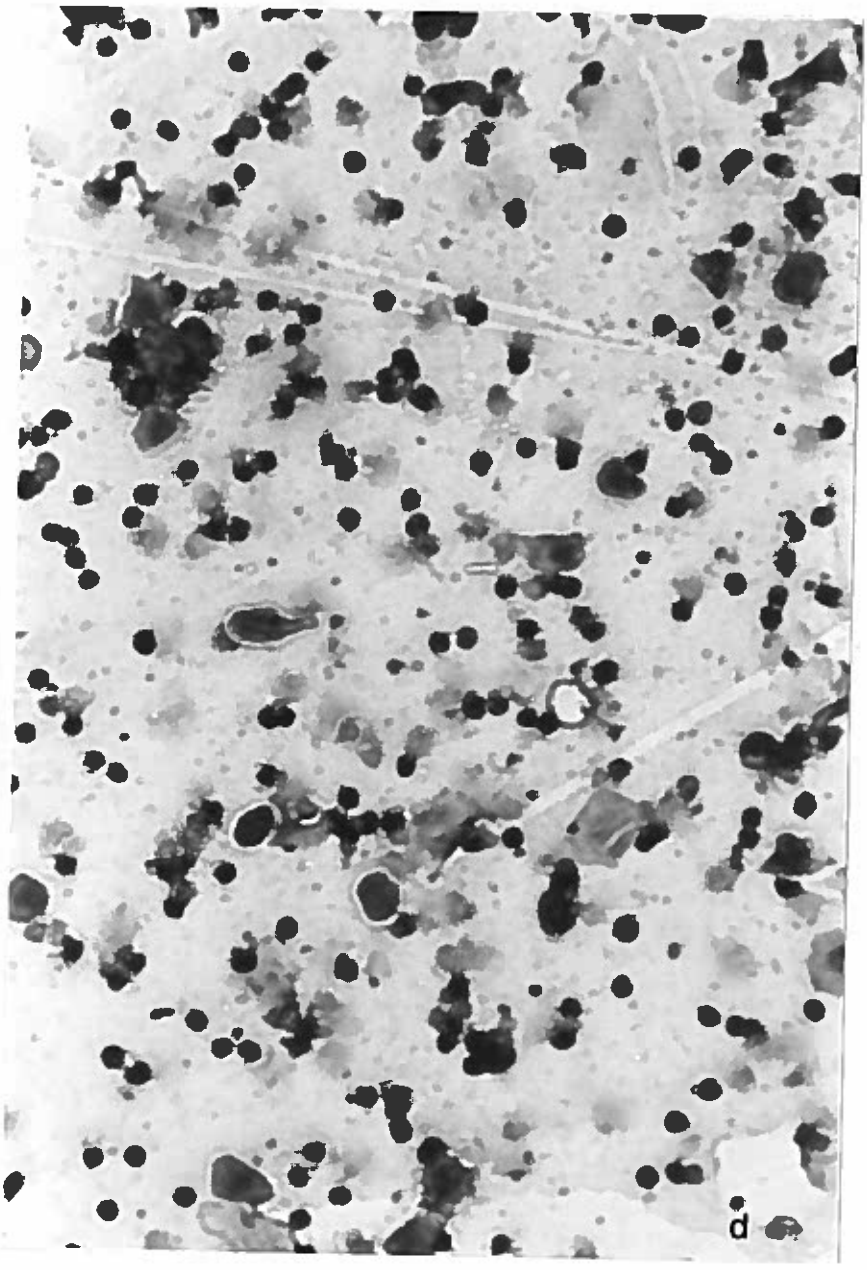

d

Fig. 7b. 30-day old rat estrous cycle for DES treated group. Estrous smear showing cornified epithelial cells predominantly.

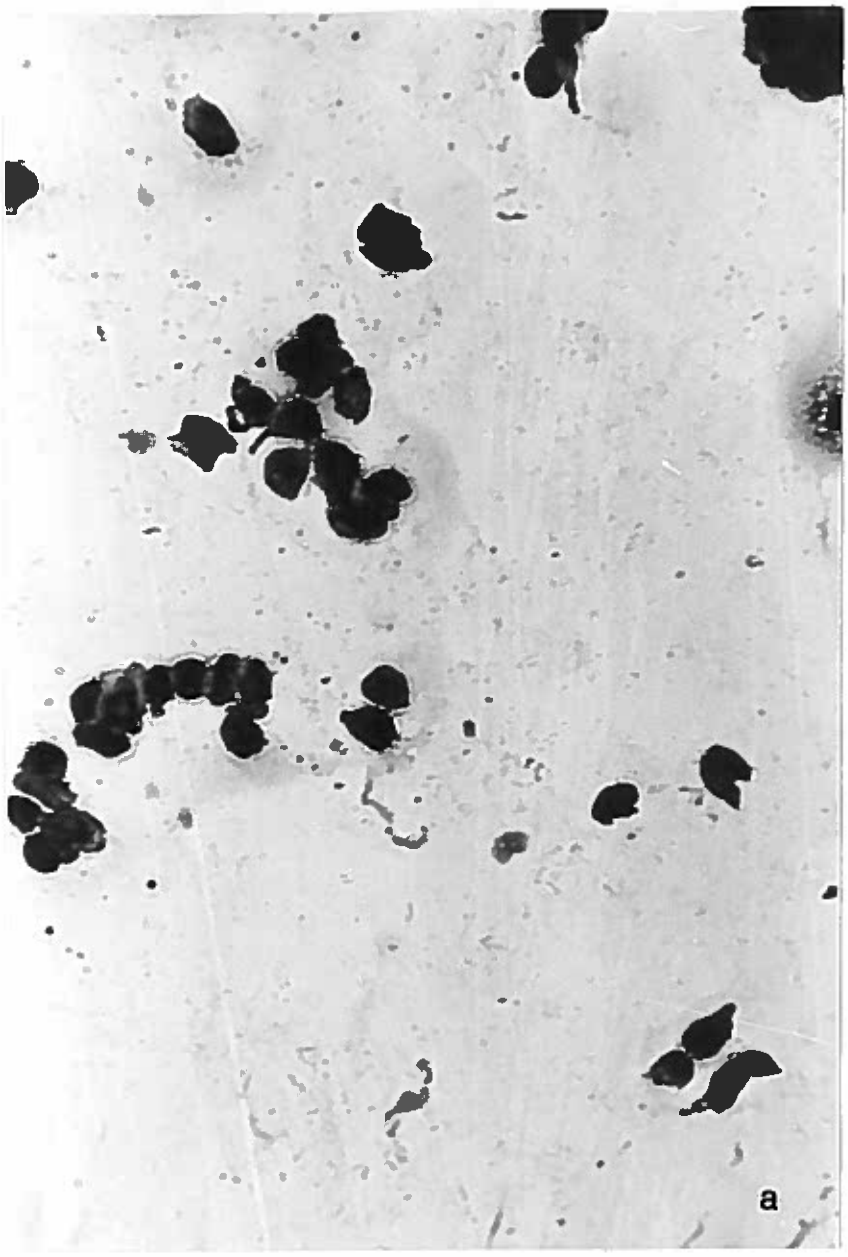

a

Fig. 7c. 30-day old rat estrous cycle for DES treated group. Met-estrous smears showing few leucocytes and cornified epithelial cells.


b

Fig. 7d. 30-day old rat estrous cycle for DES treated group. Diestrous smear showing predominantly leucocytes and few nucleated epithelial cells.

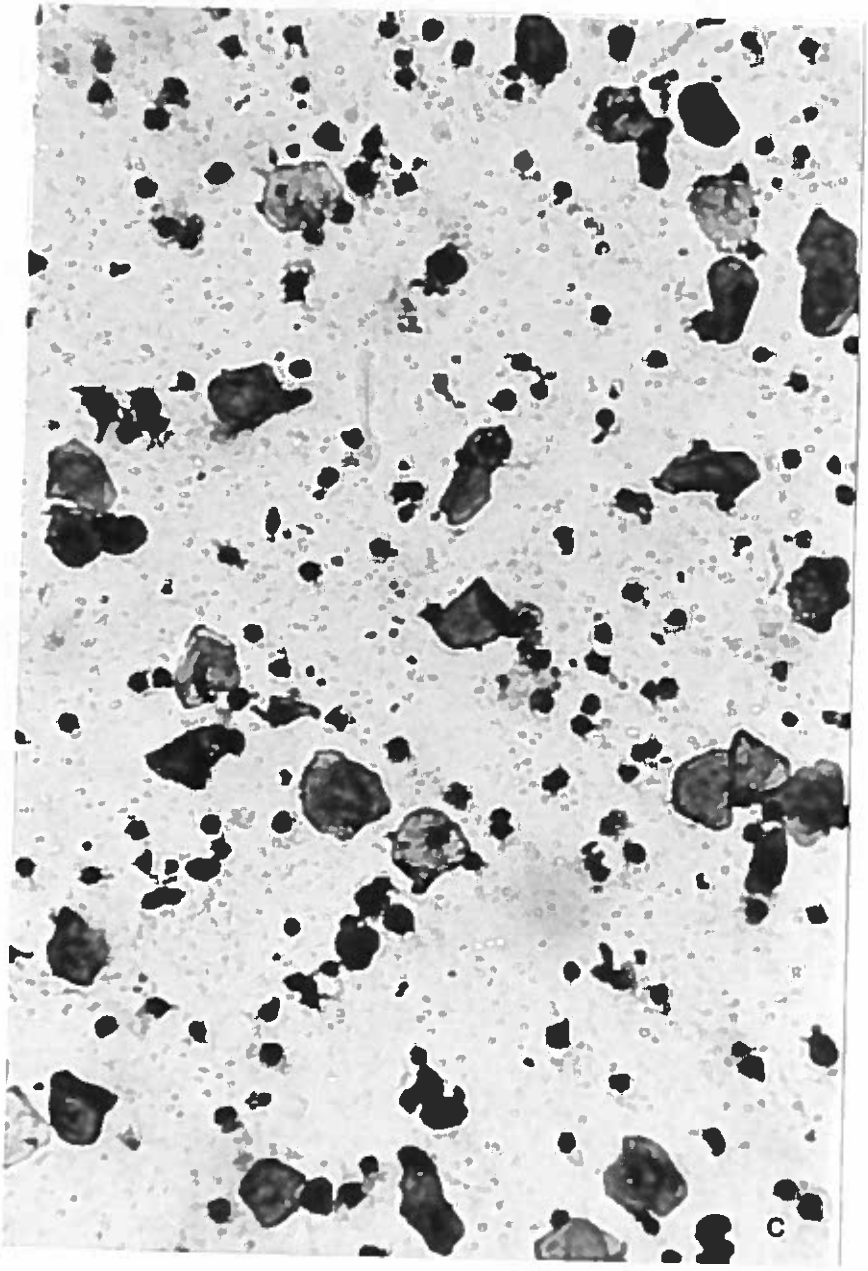
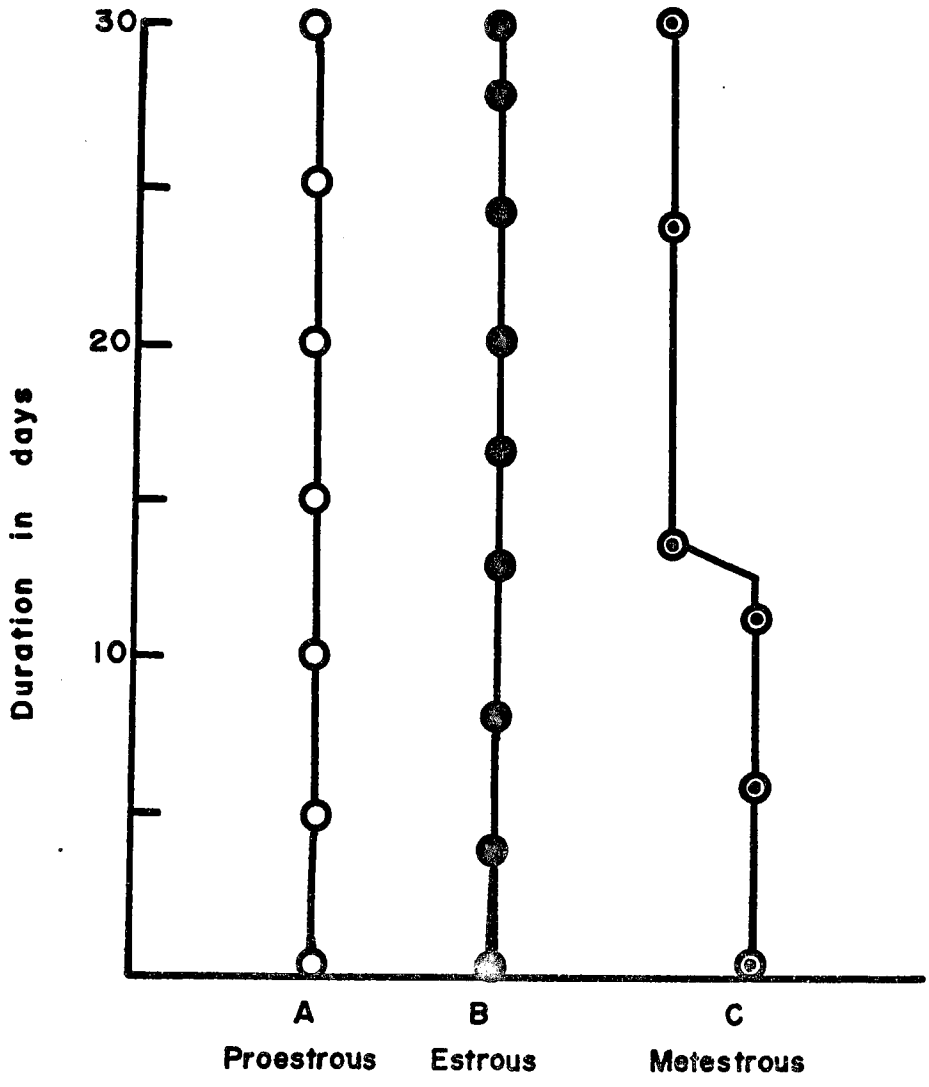

C

Fig. 8. Graph showing changes in the estrous cycle stage of 30-day old female rats to days of duration for each stage of the control, A, (○——○); saline manipulative control group (saline-treated), B, (●——●); and 35 mg DES treated group, C, (⊙——⊙).


30
20
10
Duration in days
A
Proestrous
B
Estrous
C
Metestrous

all groups (Table 1). The smears used for the slides were taken during the last ten days following treatment of 0.5 cc saline smear volume. However, no effect was noticed during this period in both the controls and the DES-treated group.

For the three groups, the cells counted for the proestrous, estrous, and diestrous stage were consistently greater than the metestrous stage for all the estrous stages of the control: 930 cells for the saline control and 220 cells for the diestrous stage of the DES treated group. The total average for the treated group was highest, with 1862 $\pm$ 256.17 (S.D.) cells counted, followed by the control with 826 $\pm$ 144.13, and 776 $\pm$ 115.86 for the saline control. The DES treated group was significantly greater at $P < 0.01$ in total average when compared to the total average of both controls (Table 1).

In the vaginal smear cell count of 30-day old female rats, the slides were prepared for each rat in the three groups as described above. However, the smear slides used for counting were taken between the 20th and 30th day following treatment. It was estimated that the number of cells counted per slide did not exceed 300 cells in 0.5 cc saline smear volume. The total average cell count was 174 $\pm$ 7.28 (S.D.) for the control, 171 $\pm$ 17.69 for the saline control, and 255.6 $\pm$ 95.77 for the DES treated group which was found to be significantly higher to both control groups at $P < 0.05$, Table 2, Fig. 9). It should be noted that reference was given only to cells present, not each type of cell typifying a particular stage of the estrous cycle.

Table 1. Vaginal smear cell count of adult female rats in 0.5 cc saline smear volume.

		ESTROUS CYCLE STAGE				
Group	Sample	Proestrous	Estrous	Metestrous	Diestrous	Total avg.± S.D.
Control	5	875	1000	665	765	826±144.13
Saline Control	5	700	930	675	800	776±115.86
35 mg DES Treated*	10	1750	1900	1600	2200	1862±256.17

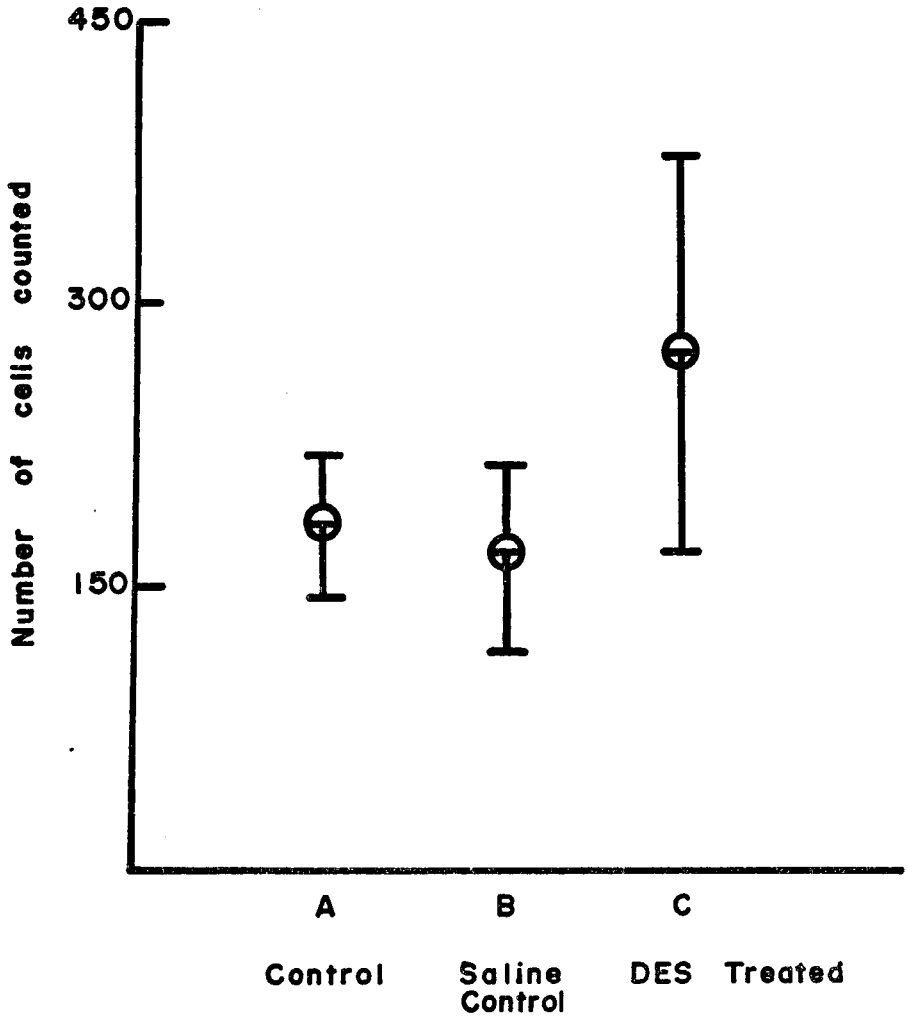
* $P < 0.01$: DES treated group compared to both control groups.

Table 2. Vaginal smear cell count of young adult female rats in 0.5 cc saline smear volume.

Group	Sample #	Total Cell Count
Control	1	174
	2	165
	3	183
	4	169
	5	179
	Tav± S.D.	174 ± 7.28
Saline Control	1	165
	2	176
	3	184
	4	154
	5	178
	Tav± S.D.	171± 17.69
DES Treated Group*	1	203
	2	210
	3	198
	4	230
	5	289
	6	240
	7	220
	8	250
	9	265
	10	270
	11	245
	12	194
	13	235
	14	290
	15	205
	Tav± S.D.	255.6 ± 95.77

* $P < 0.05$ DES treated group compared to both groups.

Fig. 9. Bar diagram showing differences in total average count of smear cells of 30-day old female rats, with standard deviations.


450
300
150
Number of cells counted
A
B
C
Control
Saline Control
DES Treated

Both ovaries of each maternal rat were utilized in the assay for each rat. The ovaries were dissected free of fat and connective tissue. The mean value of estradiol in the plasma was 14±1.58 (S.D.) pg/ml for the control, 16.4±1.82 for the saline control, and 31.2±2.62 for the DES treated group (Table 3). The increase in the treated group was significant at $P < 0.05$ level, compared to both groups of controls. The mean value of estradiol in the ovarian homogenate was 28±10.20 pg/ml for the control group, 20.2±5.40 for the saline control, and 27.5±24.55 for the DES treated group. The estradiol levels were not significantly different for the control groups (Table 3). Comparing the mean estradiol value for the plasma and ovarian homogenate in the maternal rats, there was an obvious increase in the plasma estradiol level compared to a small decrease in the mean value of ovarian homogenate estradiol value (Fig. 10). There was no difference in the mean values of both controls for the plasma values, whereas there was a change in value for the controls of the ovarian homogenate estradiol levels: 28±10.20 pg/ml for the control and 10.2±5.40 for the saline control.

At 19½-day gestation period, the ovaries of the rats are delicately small but can be visualized under a dissecting microscope. To obtain a sufficient concentration for the homogenate, all of the ovaries of the fetuses from each maternal rat were pooled. The ovaries of fetuses of maternal rats less than 19½ days were not used in the assay. The mean value of pooled ovarian homogenate estradiol levels was 7±1.87 pg/ml for the control group, 6.8±1.10 for the saline control, and 6.4±1.17 for the

Table 3. Radioimmunoassay of maternal rat plasma and ovarian tissue homogenate for E_2 level.

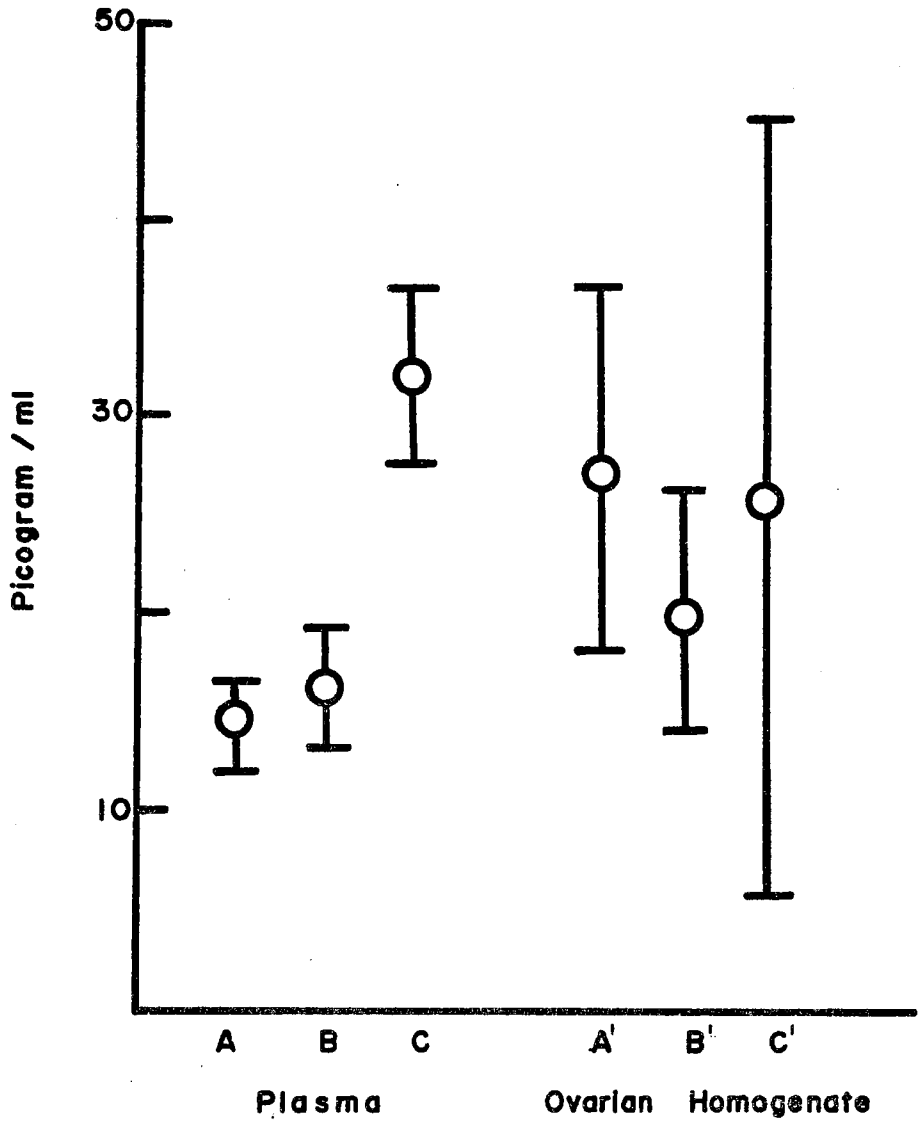
Group	Sample #	Plasma pg/ml	Ovarian Tissue pg/ml
Control	1	16	41
	2	12	23
	3	13	15
	4	14	35
	5	15	26
	Mean ± S.D.	14±1.58	28±10.20
Saline Control	1	14	14
	2	15	15
	3	17	22
	4	18	26
	5	16	24
	Mean ± S.D.	16.4±1.82	20.2±5.40
DES Treated*	1	30	30
	2	31	13
	3	35	93
	4	28	5
	5	36	17
	6	31	31
	7	28	30
	8	30	24
	9	31	18
	10	32	14
	Mean ± S.D.	31.2±2.62	27.5±24.55

* DES treated (plasma) group compared to both control groups. $P < 0.05$

Fig. 10. Diagram showing differences in mean level ± standard deviation of plasma and ovarian homogenate estradiol levels of maternal rats.

Plasma A- control, B - saline control, C - DES treated

Ovarian Homogenate A' - control, B' - saline control, C'- DES treated


50
30
10
Picogram / ml
A
B
C
A'
B'
C'
Plasma
Ovarian Homogenate

DES treated group (Table 4). These values show no difference in all three groups compared. The mean value for estradiol in the plasma of 30-day old rats was 31±2.35 pg/ml for the control, 32.8±1.92 for the saline control and 23.4±3.94 for the DES treated group (Table 5). There was a significant decrease in mean estradiol value of the treated group when compared to both control groups at $P < 0.1$ level. The ovarian homogenate estradiol level in pg/ml was 18.6±3.29 for the control, 18.4±4.83 for the saline control, and 20±6.2 for the DES treated group. There was no significant difference in both the treated and the control group for estradiol levels. Comparing the plasma and ovarian homogenate estradiol values, there was a decrease in plasma estradiol level and a slight difference in ovarian homogenate estradiol value, compared to the control values (Fig. 11).

Table 4. Radioimmunoassay of 19½ day old pooled ovarian tissue homogenate for E_2 level.

Group	Sample #	Ovarian Homogenate pg/ml
Control	1	8
	2	9
	3	5
	4	8
	5	5
	Mean ± S.D.	7±1.87
Saline Control	1	8
	2	7
	3	5
	4	7
	5	7
	Mean ± S.D	6.8±1.10
35 mg DES Treated	1	8
	2	5
	3	5
	4	6
	5	8
	6	7
	7	7
	8	6
	9	5
	10	7
	Mean ± S.D.	6.4±1.17

Table 5. Radioimmunoassay of 30 day old female rat's plasma and ovarian homogenate for E_2 level.

Group	Sample #	Plasma pg/ml	Ovarian Homogenate pg/ml
Control	1	30	18
	2	29	22
	3	31	15
	4	30	22
	5	35	16
	Mean ± S.D.	31±2.35	18.6±3.29
Saline Control	1	30	23
	2	33	24
	3	32	13
	4	34	16
	5	31	16
	Mean ± S.D.	32.8±1.92	18.4±4.83
DES Treated*	1	24	14
	2	22	16
	3	23	16
	4	26	31
	5	22	13
	6	21	25
	7	33	14
	8	20	26
	9	19	15
	10	23	24
	11	24	22
	12	25	23
	13	30	30
	14	19	13
	15	20	19
	Mean ± S.D.	23.4±3.94	20±6.2

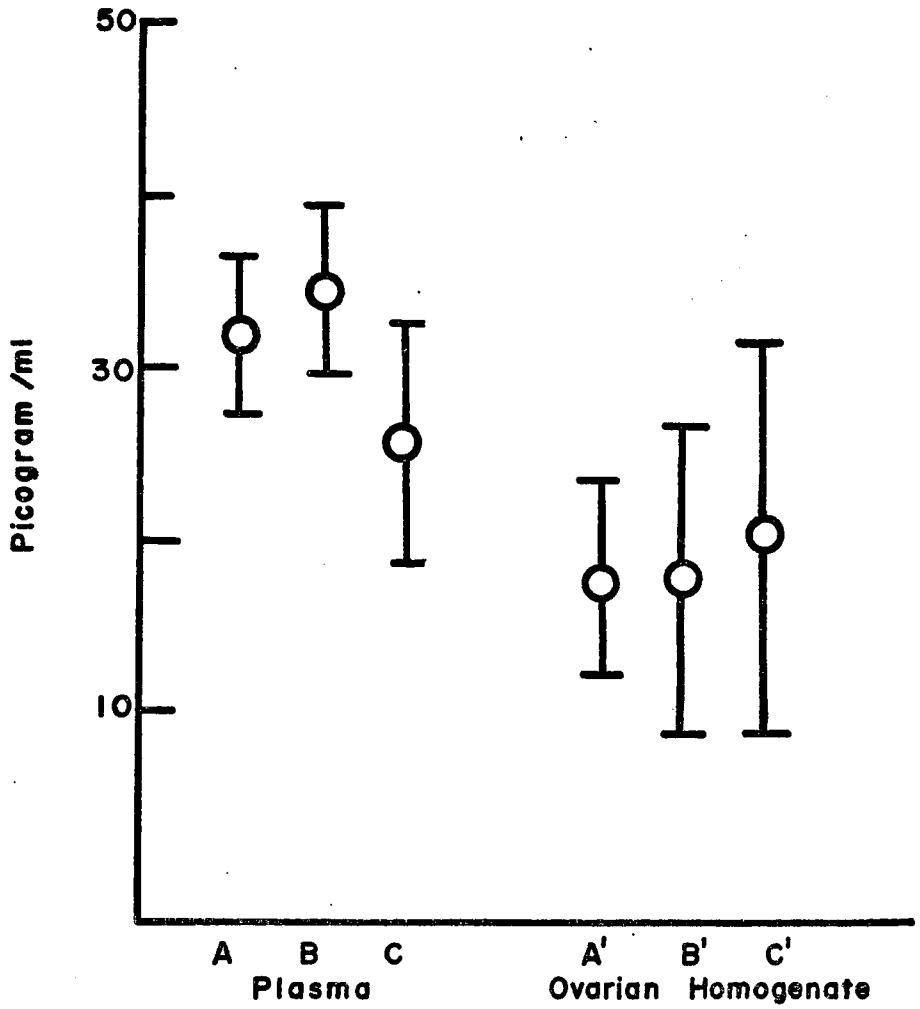
* DES treated (plasma) group compared to both control groups (Nonsignificant) $P < 0.1$

Fig. 11. Diagram showing the differences in mean value ± standard deviation of plasma and ovarian homogenate estradiol levels of 30 day old female rats.

Plasma A - control, B - saline control, C - DES treated

Ovarian Homogenate A' - control, B' - saline control,

C' - DES treated


50
30
10
Picogram /ml
A
B
C
A'
B'
C'
Plasma
Ovarian Homogenate

CHAPTER V

DISCUSSION

The present study has demonstrated that administration of diethylstilbestrol (35 mg/2 cc/kg body weight) does not alter the estrous cycle synchrony of the adult female rats. This is consistent with the view that synthetic estrogen has no effect presently on the maternal host but very much so on their offsprings. It can thus be speculated that the DES was cleared from the rat's ysstem so quickly that it did not have time to have an effect on their estrous cycle synchrony. When labelled mestranol (M) is administered to rats, it is demethylated and the resulting ethynylestradiol (EE_2) is concentrated in the uteri. This led to the postulate that M might be active only after demethylation. Bird and Clark (1973) were interested in the metabolism of these steroids in humans and the possibility that EE_2 might be found in the blood following M administration. They concluded that the principal circulating form of both synthetic estrogens appears to be ethynylestradiol sulfate. This has therefore shed light on the idea that estrogen effects were manifested in the offspring and, according to Naftolin, et al. (1971), androgens produced their effect by being converted to estrogen at a cellular site thus exposing the possibility of a cellular genetic effect. Moreover, hormones are known to affect gene action at the transcriptional level (McKern, 1969; Malley, et al., 1975). Observations on DES treated 30-day old female rats, however, present a different picture. The administration of this synthetic estrogen does have a significant effect on the estrous cycle of the young female rat.

These rats remain in persistent estrous or proestrous after treatment, with the onset of the effects taking place at different days. The changes observed in the treated group are valid in that neither the saline manipulative control nor the control showed any change in estrous cycle synchrony. According to Turner and Bagnara (1976), female rats treated with estrogen from the day of birth to 30 days do remain in persistent diestrous and later become sterile adults. This study confirmed current observation, except that in studies of the DES treated virgin female rates, they were found to remain in persistent estrous or proestrous regardless of the stage in the cycle at which they were treated with DES. Moreover, the onset of effect occurred between the 5th, 10th or 13th day.

The persistent estrous or proestrous observed in these studies indicates a high proliferation of cells manifested as a secondary effect of what is the actual primary effect of stilbestrol on the reproductive tract. Anderson and Kang (1975) stated that estrogen enhances proliferation and keratin formation in the cervico-vaginal epithelium of immature rats, whereas the estrogen anatagonist, Cl-628, represses mitosis and induces mucinogenesis. Iguchi, et al. (1976), however, reportted that the vaginal epithelium showed estrogen dependent proliferation and parakeratosis in mice given estradiol-17B for 5 days, beginning on the day of birth and killed a few days after the treatment. In contrast, they found that in neonatal mice treated with 30 mg/E for 5 days, the vaginal epithelium exhibited estrogen-independent proliferation and cornification or parakeratosis.

Treatment of female mice with estrogen during neonatal life induces two different types of persistent proliferation and cornification or parakeratosis of the vaginal epithelium in adulthood. Takasugi, et al. (1970) suggested that one type of persistent vaginal change brought about by low doses of estrogen, may be ascribed to a permanent alteration of the hypothalomo-hypophseal system and is directly related to a continued secretion of ovarian estrogen. The other type, elicited by high does of estrogen, is due to permanent changes in the vaginal epithelium itself and is estrogen independent. From these estrogen independent vaginal changes, Mori (1969) found that cancerous or precancerous lesions frequently developed in advanced age. These studies thus suggest a loss of estrous cycle synchrony after treatment of the virgin female Long-Evan rats with DES.

Questions can be raised from findings of several workers like Harris (1964) who showed that the hypothalamus of the rat at birth is undifferentiated with respect to sex and can thus be modified by exogenous testosterone or estrogen. This with previous mentioned data suggested that endogenous steroid synthesis occurs shortly after birth to complete sexual differentiation of the hypothalamus. Also, since in the absence of a testis, a female pattern of gonadotropin release persists, a passive role for the ovary during this period could be inferred. However, from histochemical observations (Presl, et al., 1965), the measurement of unilateral ovarian morphology (Reiter, et al., 1972), there is evidence for estrogen synthesis before 10 days post partum. In the 30-day old female rat, estrous

cycle synchrony was manifested for about 3 weeks before treatment, disputing the fact that the hypothalamus is undifferentiated in this young adult rat, thus having an effect leading to the asynchronous estrous cycle. Furthermore, the asynchronous cell proliferation in vaginal epithelium, leading to prolonged specific stages of the estrous cycle can in part be explained according to Lu, et al (1977). They reported that the positive feedback of estrogen and progesterone on LH release is reduced in old as compared to young cycling female rats, and is believed to account at least in part for the irregular cycling and failure of ovulation in the old constant estrous rats.

The data obtained from the vaginal smear cell counts of adult and 30-day old female rats substantiate the hypothesis that dielthylstilbestrol enhances high cell proliferation of vaginal epithelium. The high cell count of the adult rat at estrous which was higher compared to that of other stages of the estrous cycle thus show a heightened estrogen action leading to proliferation of the female reproductive tract, a well known estrogen effect. The high cell count obtained at diestrous stage in the treated group of maternal rats (220) compared to the two controls (765 control, and 800 saline control) might be due to heightened secretion of estrogen on diestrous-2, as reported by Freeman, et al. (1976) and Neill, et al. (1971). This is required for the release of an ovulatory amount of LH on the following day. Proestrous can effect the proliferation of the vaginal epithelium also.

The 30-day old female rat smear counts showed a significant increase

in the DES treated group (255.6±95.77) compared to the control (171±17.09). These values show increased estrogen action on the vaginal epithelium, leading to asynchronous cell proliferation. The results further support the findings of Anderson and Kang (1975) that estrogen enhances proliferation and keratin formation in the cervico-vaginal epithelium of immature rats and of Iguchi (1976) that high doses of estrogen in C57 Black/Tn mice leads to estrogen independent proliferation and cornification of vaginal epithelium.

Is the high cell count obtained, estrogen dependent or estrogen independent? The works of several workers have pointed out that low does of estrogen do cause persistent proliferation of vaginal epithelium, as do high doses of estrogen (Iguchi, et al, 1976; Takasugi, et al., 1970). It was Takasugi, et al. (1970) who suggested that the one type of persistent vaginal change brought about by low doses of estrogen is ascribable to a permanent alteration of the hypothalomo-hypophyseal system and is directly related to a continued secretion of ovarian estrogen. The other, elicited by high doses of estrogen, is due to permanent change in the vaginal epithelium itself and is estrogen-independent. The counts obtained in this study can thus be attributed to an estrogen-independent phenomenon.

Fotherby and James (1973) have described in detail the metabolism of synthetic estrogens in the rat. The existing situation then was that only small amounts of the dose were recovered within a 24 hr period. More recent studies have been conducted and yet there is no definitive answer. In the case of ethynylestradiol, 3.9% was found in the urine and 5.5% in

the feces. The corresponding values for the cyclopenyether of ethynyl-estradiol were 5.9% and 7.3% respectively. Large amounts of the dose were found in the bile and most of the radioactivity in plasma was present as sulfate conjugates. Since the ovary is the major source of estrogen synthesis, the study was aimed at elucidating the idea of whether DES has any effect, inhibitory or enhancement, on the ovarian estrogen level in the maternal, fetal and young adult female rats.

Measurement of estradiol levels in the plasma and ovarian homogenates showed no significant difference in the maternal rat two days before parturition. However, there was a significant increase in the plasma level of the treated group compared to the two controls. Also, there was no significant difference in the ovarian homogenate estradiol level of the fetus. There was, however, a high increase difference in the estradiol level of maternal ovarian homogenate compared to that of the fetuses. These findings are compatible with those of Presl, et al. (1969) and Shaikh and Abraham (1969) who reported a gradual decline in plasma estradiol level as pregnance nears termination. Preedy and Aitkens (1958) demonstrated a lower fetal estradiol level than maternal. The estrogens present in fetal blood are represented mainly by estriol, whereas those of the maternal blood and placenta contain considerable amounts of estrone and estradiol-17B, in addition to estriol. This seems to indicate that the fetus is capable of rapidly metabolizing estrone and estradiol. Further, because of the higher concentration of estrone and estradiol in maternal plasma, there may be a tendency for these hormones to diffuse in the

reverse direction, that is, from maternal to fetal plasma. Preedy and Aitkens further proposed that direct comparison between steroid concentrations in maternal and fetal circulation is complicated in the case of estrogens by the unknown distribution in plasma between "free", "protein bound", and "conjugated" forms. The data for the estradiol level in ovarian homogenates of the present study show that estrogen synthesis in the fetal ovary is minimal and there seems to be no evidence of DES enhancing or inhibiting it.

The high increase in plasma estradiol level of the DES treated maternal rats supported the work of Longscope, et al. (1974) that the use of mestranol appears to have little effect on endogenous estrogen metabolism while that of ethynyl estradiol appears to increase the metabolic clearance rate of estradiol. On the other hand, the insignificant change in ovarian homogenate and plasma estradiol levels differs from Gallegos and Gallegos (1975) who reported a higher estradiol tissue concentration than estradiol peripheral plasma concentration in the human female reproductive tract tissues. Moreover, the females studies were not pregant, while the rats in this study were pregnant.

One characteristic of pregnancy in primates is the high level of estrogen in plasma and urine (Beling, et al., 1973). Most of the estrogen in human primates is from the placenta which contains an abundance of enzymes which convert it to andogens according to Beling. In partial agreement with the results obtained, Resko, et al. (1975) observed that though large amounts of estradiol can be quantified in the systemic

circulation of the mother, very little is found in the fetal circulation. The reason is not clear but it may be due to the rapid conversion of estradiol (E_2) to some other compounds in the fetus but not in the mother. Estrone (E_1) may be the major secretory product of the placenta of the rhesus monkey. In the fetus, the small amounts of E_2 may be derived entirely from E_1 by the action of dehydrogenases at peripheral sites. The fact that estrone and estradiol of fetal origin are significantly correlated, points to this possibility. It is known, however, that more E_2 than E_1 is found, either because E_1 may be converted very rapidly to E_2 in the mother or because there is some other source of E_2 in the mother, such as the ovary or the adrenal gland. Thus it is probable that both mechanisms operate in the maternal compartment. Resko, et al. (1975) concluded that the major source of fetal estrogen in the rhesus monkey is the placenta because significantly larger quantities of estrogen were found in the umbilical veins than in the umbilical artery.

The estradiol level in the plasma of treated asynchronous estrous cycle 30-day old female rats showed a significant decrease when compared to the controls. There was no significant difference in the estradiol level in the plasma and ovarian homogenate. However, there was a significant difference in the estradiol levels of the plasma and ovarian homogenate of the two controls. The speculation is therefore warranted that DES has a decreased effect on the plasma level and no effect on the ovarian homogenate level. This result is in agreement with that of other workers like Hendricks, et al. (1971), Gomer, et al. (1977), and Smeaton, et al.

(1975). However, Resko's (1977) data on rhesus monkey ovaries before ovulation suggested that progesterone (PO) lowers estradiol (E_2) and testosterone (T) in the systemic circulation before ovulation by other mechanisms since P does not exert direct inhibitory effects on the biosynthesis of E_2 and T by the preovulatory ovary *in vivo*.

According to Smeaton, *et al*. (1975), changes in gonadal activity occurring with increasing age are probably relevant to the intact animal. In female rats, the peak in estradiol-17B synthesis at day 10 coincides with the time at which exogenous steroids were found to lose their effect on the hypothalamus (Barraclough, 1976). It is therefore reasonable to propose that *in vivo* a short period of estradiol-17B synthesis can imprint the hypothalamus with an irreversible female pattern of gonadrotropin secretion, or affect some content in the brain to make it refractory to steriods or their derivatives. An alternative, according to Smeaton, *et al*. (1975), is that a gonodotropin (FSH) is stimulating the ovaries to produce estrogen, but this is incidental to an imprinting effect of the gonadotropin on the hypothalamus by a short feedback loop.

The results thus show that even though DES does not have any effect on estradiol level synthesis in the ovary, it does hav an effect in stimulating the ovary to induce ovulation at low doses. At high doses, it interferes with ovulation due to the negative feedback of estrogen on the hypothalamo-hypophyseal axis, thereby inhibiting ovulating hormone (Ying and Greep, 1971). This was manifested by the asynchrony of estrous cycle and increased cell count obtained for the treated rats; but,

contrary to the work of Meyer and Bradburg (1960), who reported that large doses of stilbestrol stimulate effective amounts of estrogen in the ovaries, no difference in estradiol level of ovarian homogenate in both the controls and treated group was demonstrated in the present study.

It is known that in the early part of the menstrual cycle, the synthesis and secretion of estrogen are due primarily to FSH stimulus of the theca internal cells. Also, Gay, et al. (1970) reported that FSH release may be partly dependent on secretion of estrogen and other steroids since FSH shows increased output at about the same time. Therefore, the decrease in plasma level may be attributed to mechanisms other than DES' effect at the pituitary level. Goomer, et al. (1977) concluded that testosterone propionate and estradiol-17B have a direct negative feedback influence on the hypothalamus in the neonatal female rats; alter the normal pattern of plasma and pituitary LH in developing female rats, and prevents the cyclic secretion of plasma LH after maturity. Shaikh (1971) suggested that progesterone may influence the decrease in estrogen secretion, perhaps by inhibiting further gonadotropin output or by direct action on the ovary. This present study clearly shows that there is no difference in the ovarian estradiol level of the treated and the control rats. There is, however, a significant decrease in the plasma estradiol level, resulting probably from DES' effect on the pituitary level rather than on the ovary itself.

CHAPTER VI

SUMMARY

The present study demonstrated that administration of 35 mg of diethylstilbestrol (DES) has no effect on the estrous cycle synchrony of the adult female Long-Evans rats.

In the 30-day old Long-Evans female rats, however, the administration of 35 mg diethylstilbestrol has a marked and significant effect on their estrous cycle. The young non-gravida rats remained in persistent estrous or proestrous after DES treatment. The onset of these observed effects occurred between the 5th, 10th and 13th day after treatment. Since the oviduct and the vaginal tract in previous studies have been suggested as the primary target organ for synthetic hormone activities, these data now report a secondary effect - asynchronous cell proliferation in vaginal epithelia leading to prolonged specific stages of the estrous cycle.

A significant increase in cell counts of vaginal smears in 0.5 cc saline smear volume was obtained in both the treated groups of the adult and 30-day old young Long-Evans rats compared to the controls of both age groups.

With radioimmunoassay, estradiol levels in the plasma and ovarian homogenate showed no significant difference in the maternal rats. However, there was a significant increase in the plasma level of the treated group compared to the controls.

There was no significant difference in the ovarian homogenate estradiol level of the maternal fetuses; however, there was a high increase in

the estradiol level of the maternal ovarian homogenate compared to that of the fetal estradiol level in ovarian homogenate.

Lastly, the estradiol level in the plasma of the DES treated 30-day old young female rats showed a significant decrease compared to the controls. However, no significant difference was noticed in the estradiol levels in the plasma and ovarian homogenate of the treated rats.

It can thus be concluded that DES has no direct effect on the estrogen level of the ovarian homogenate of the maternal, fetal and 30-day old young Long-Evens female rats.

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